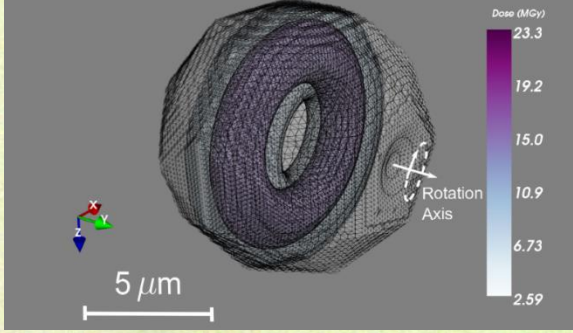
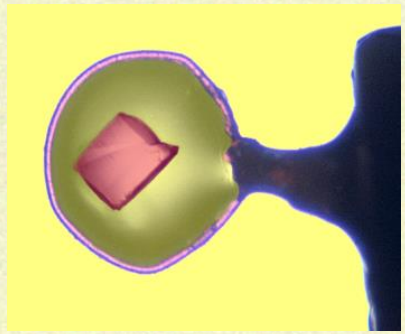
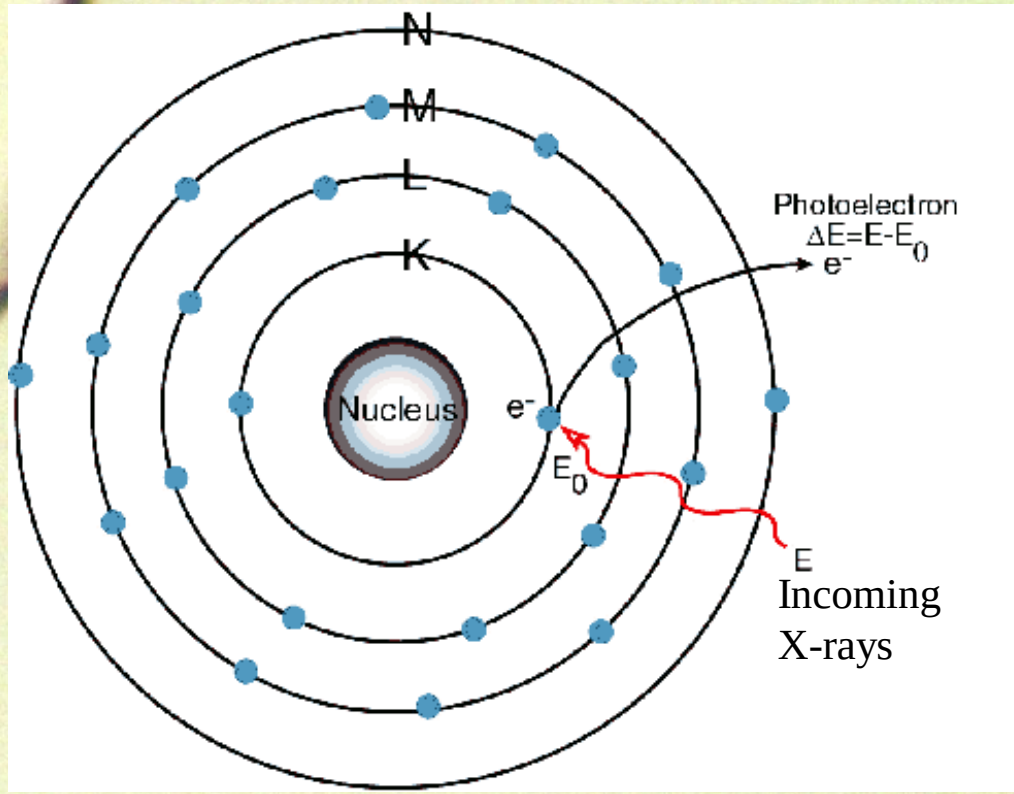
RADIATION DAMAGE:

why do we care?

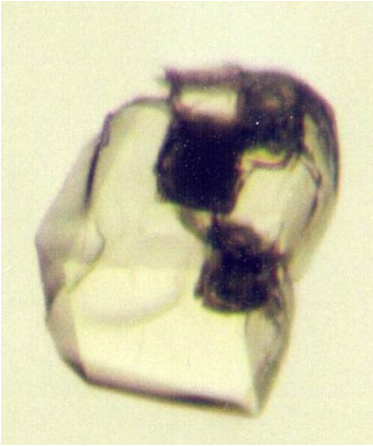


CCP4/DLS Workshop,
29th November 2021





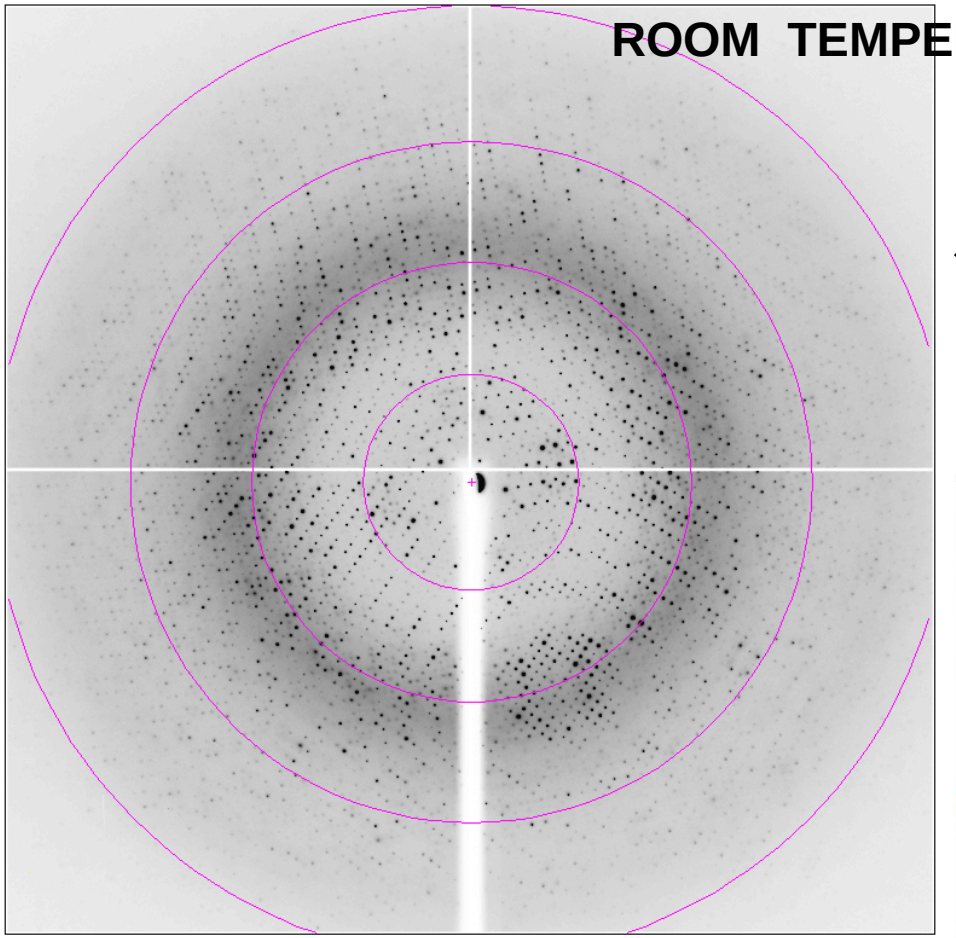
Elspeth.garman@bioch.ox.ac.uk



Radiation damage:
The Plan:

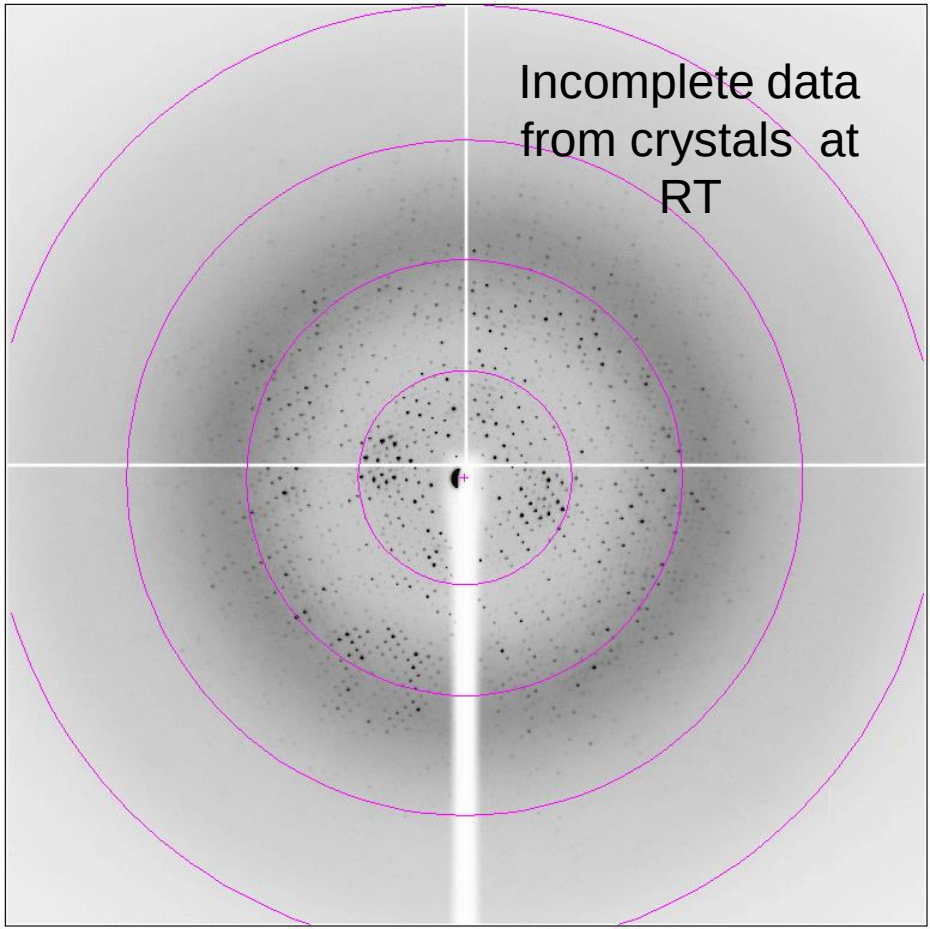
- **What are the symptoms?**
- What is it?
- Why do we care? Effect on MAD/SAD.
- How do we calculate the Dose?
- What do we know/would like to know?

ROOM TEMPERATURE

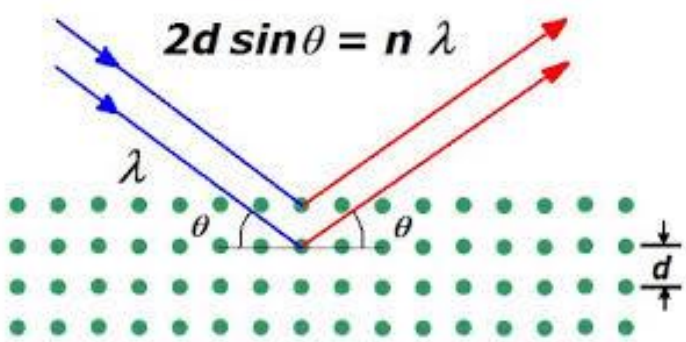


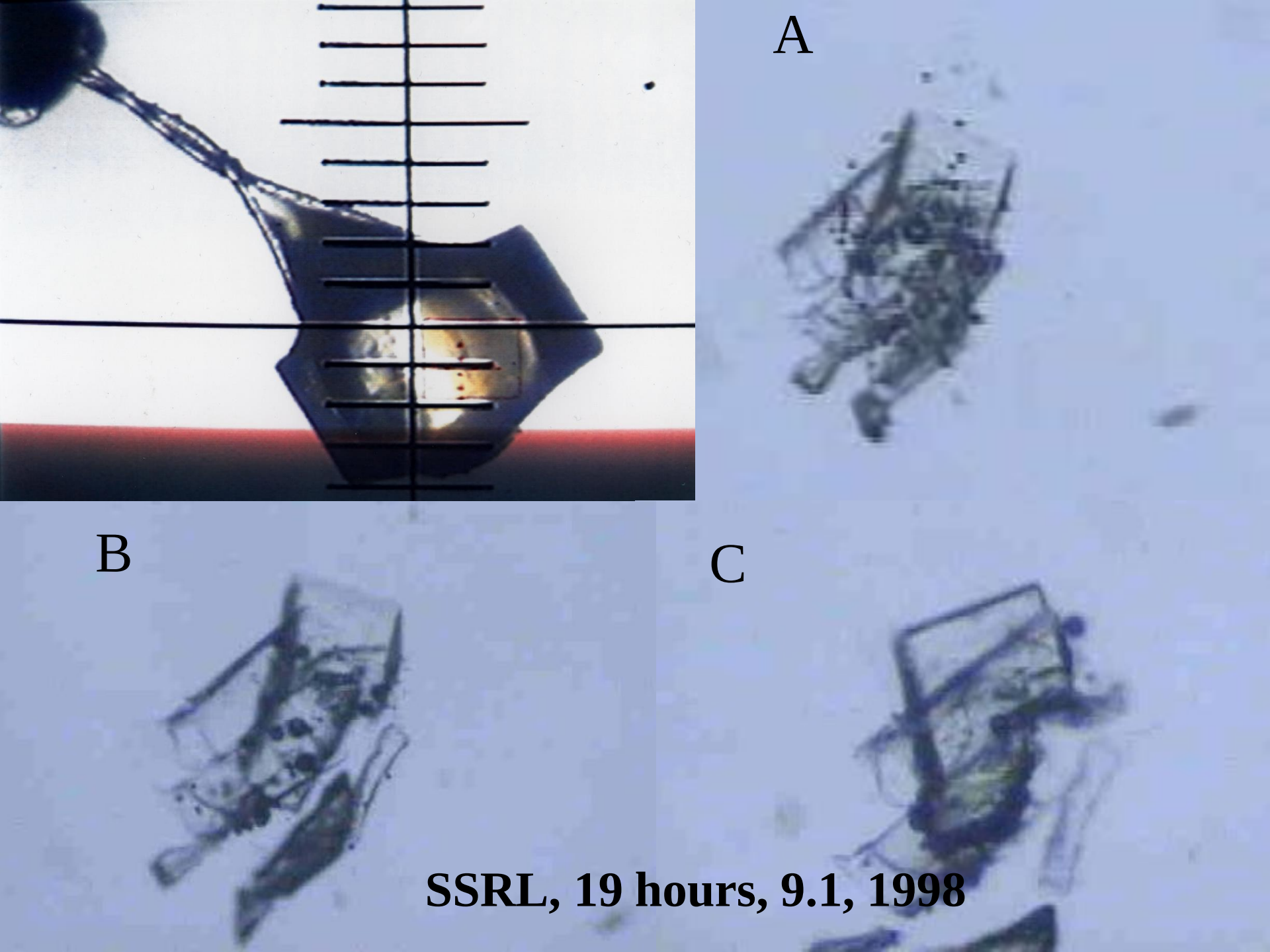
Intensity decrease with dose

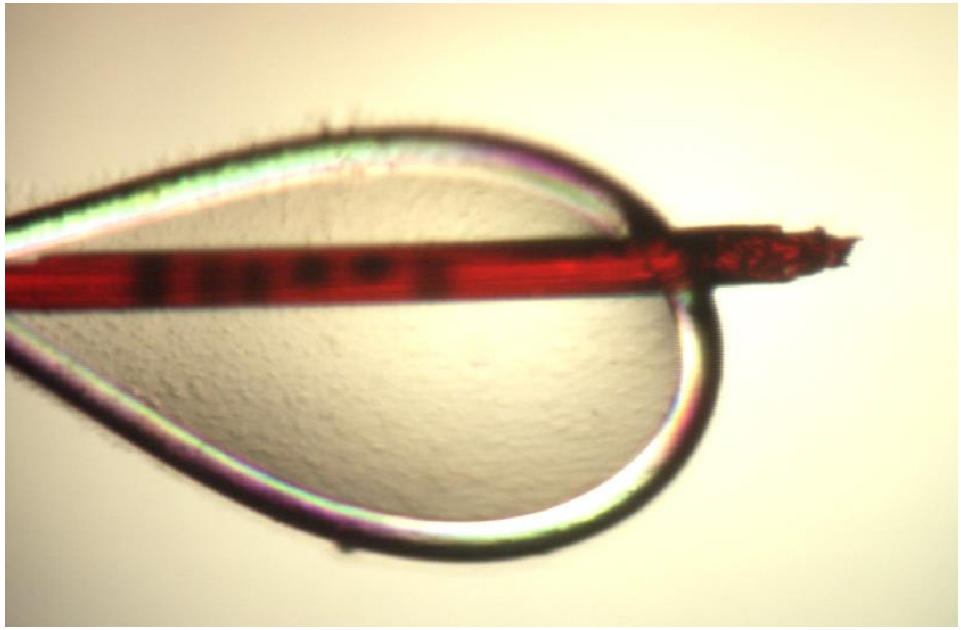
Loss of diffraction



Incomplete data from crystals at RT

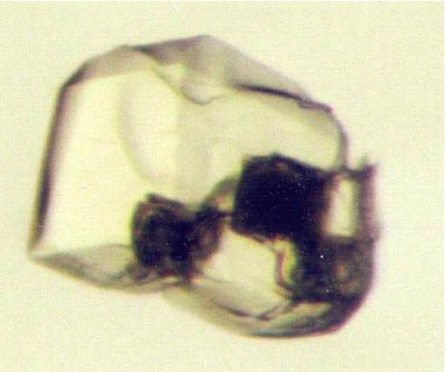


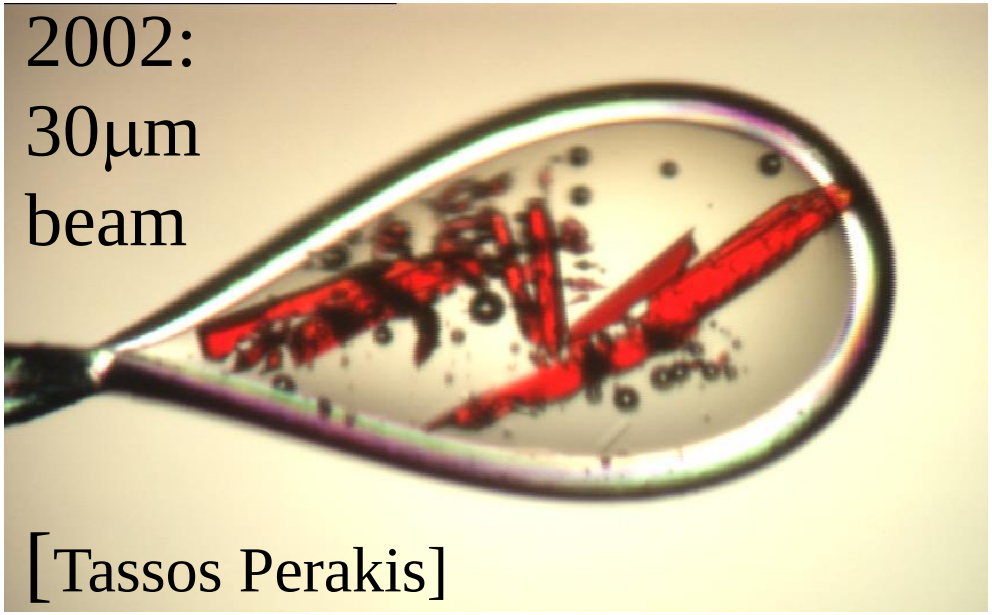

A
B
C
SSRL, 19 hours, 9.1, 1998



1995 onwards: 100 K

BUT THEN, 1999:

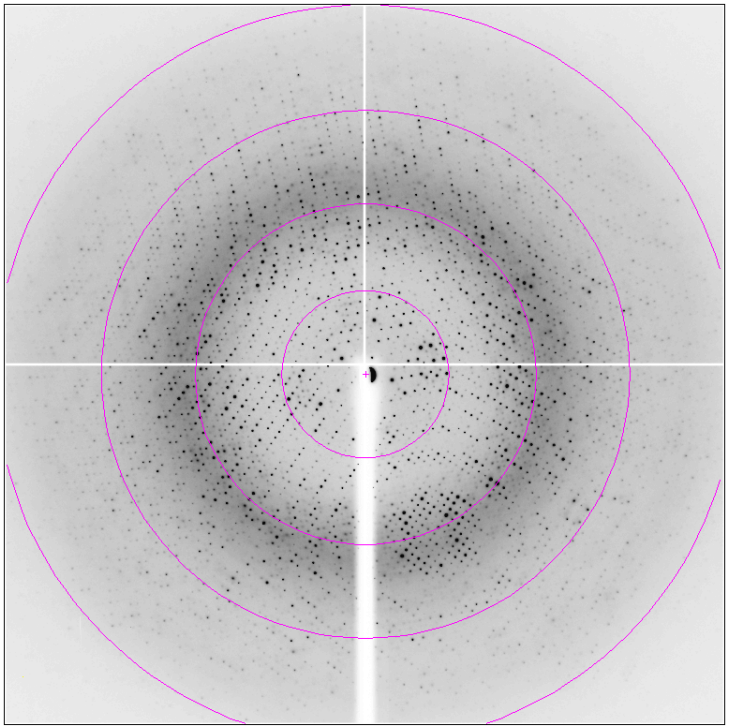




Also observe spectral changes

Iron containing protein, ESRF

Garman & Owen (2006), Acta D62

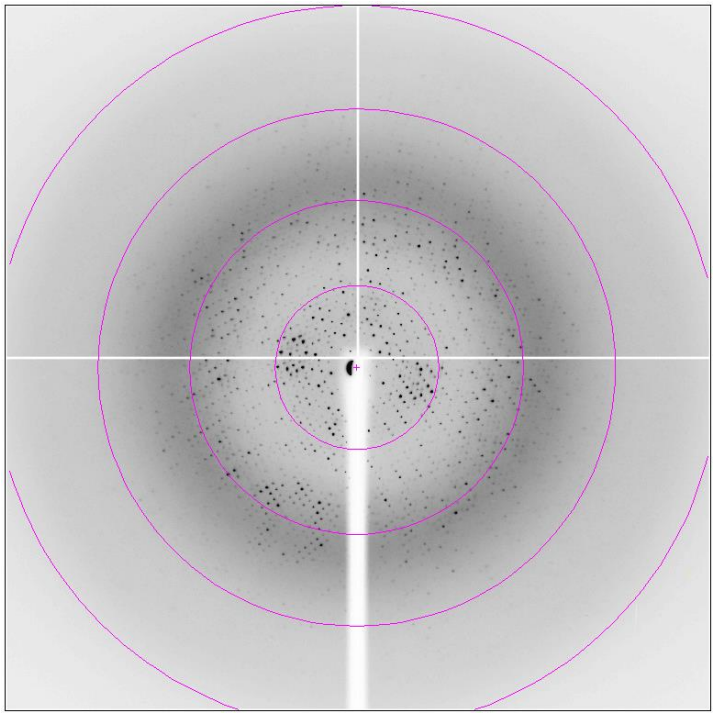


At 100 K

Intensity decrease

Loss of diffraction

Incomplete data from crystals



Happens during 1 dataset at 100K for many crystals

Unit cell volume expansion

Increase in Wilson B factors, Rmerge

Increase in mosaicity

'GLOBAL' damage

ESRF 2000:

1×10^{12} ph s^{-1} into 100μm square slits

Diamond Light Source:

3×10^{12} ph s^{-1} into 7μm×6μm

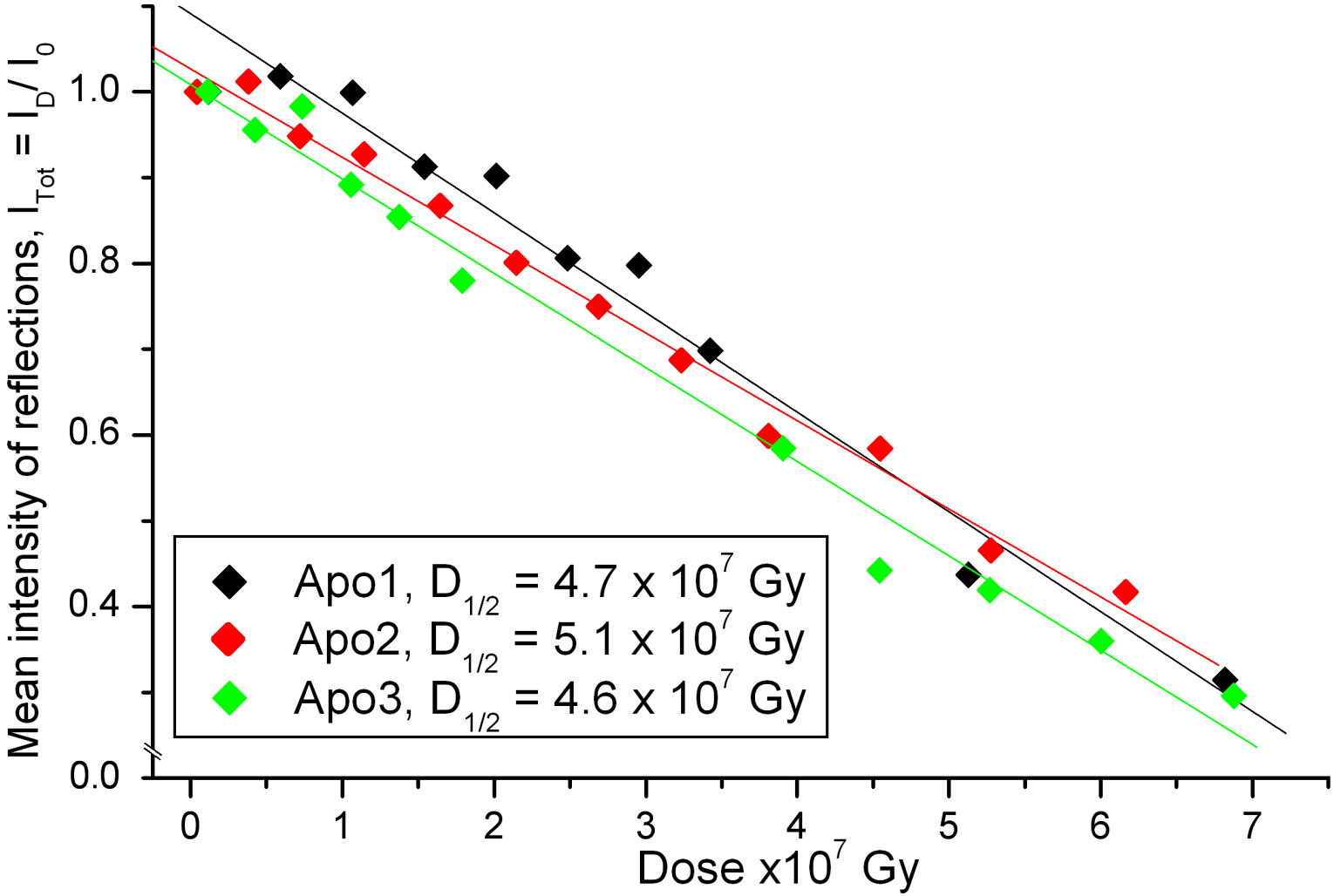
[7×10^{14}]

Australian synch.

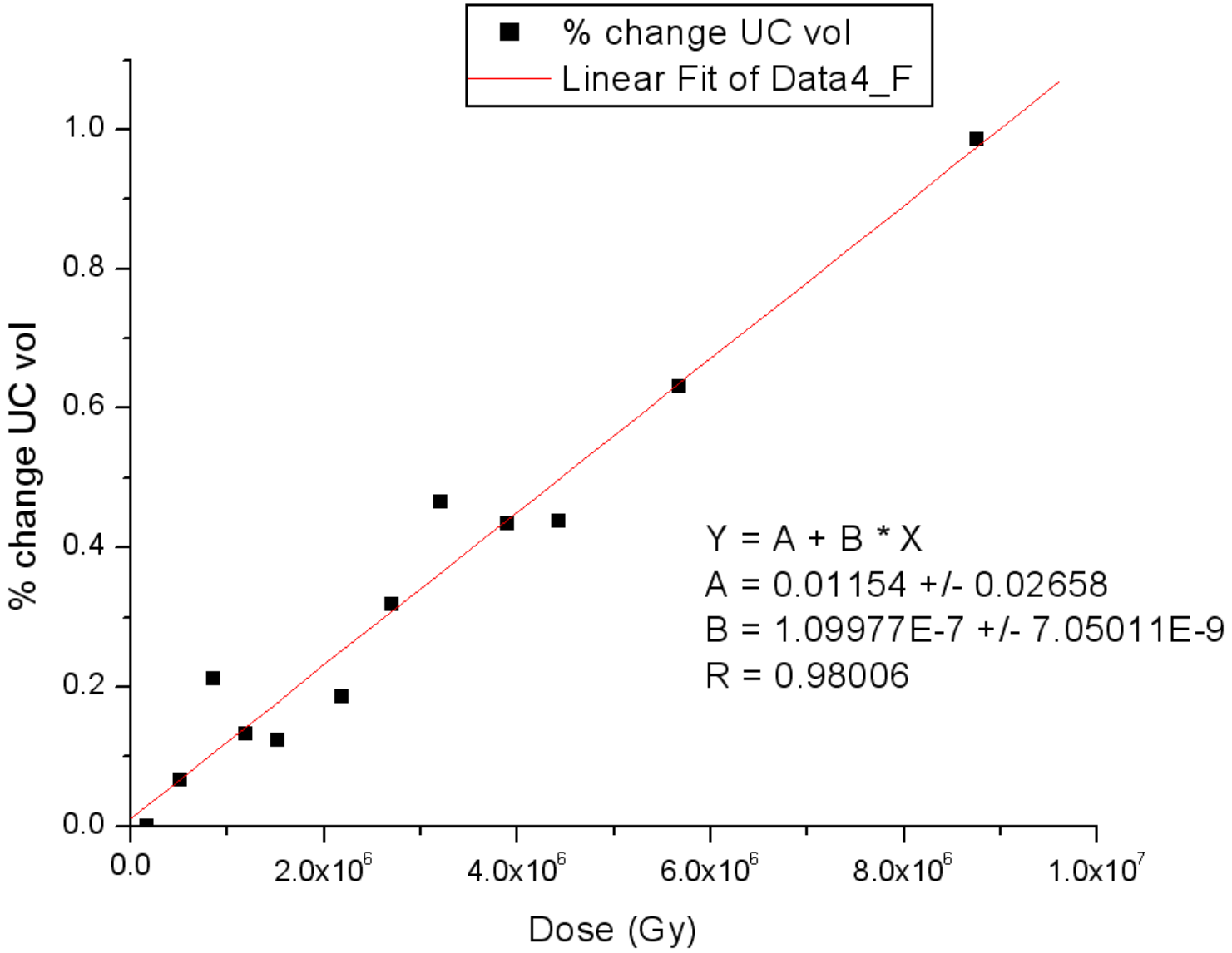
3×10^{13} ph s^{-1} into 50μm × 70μm [10^{14}]

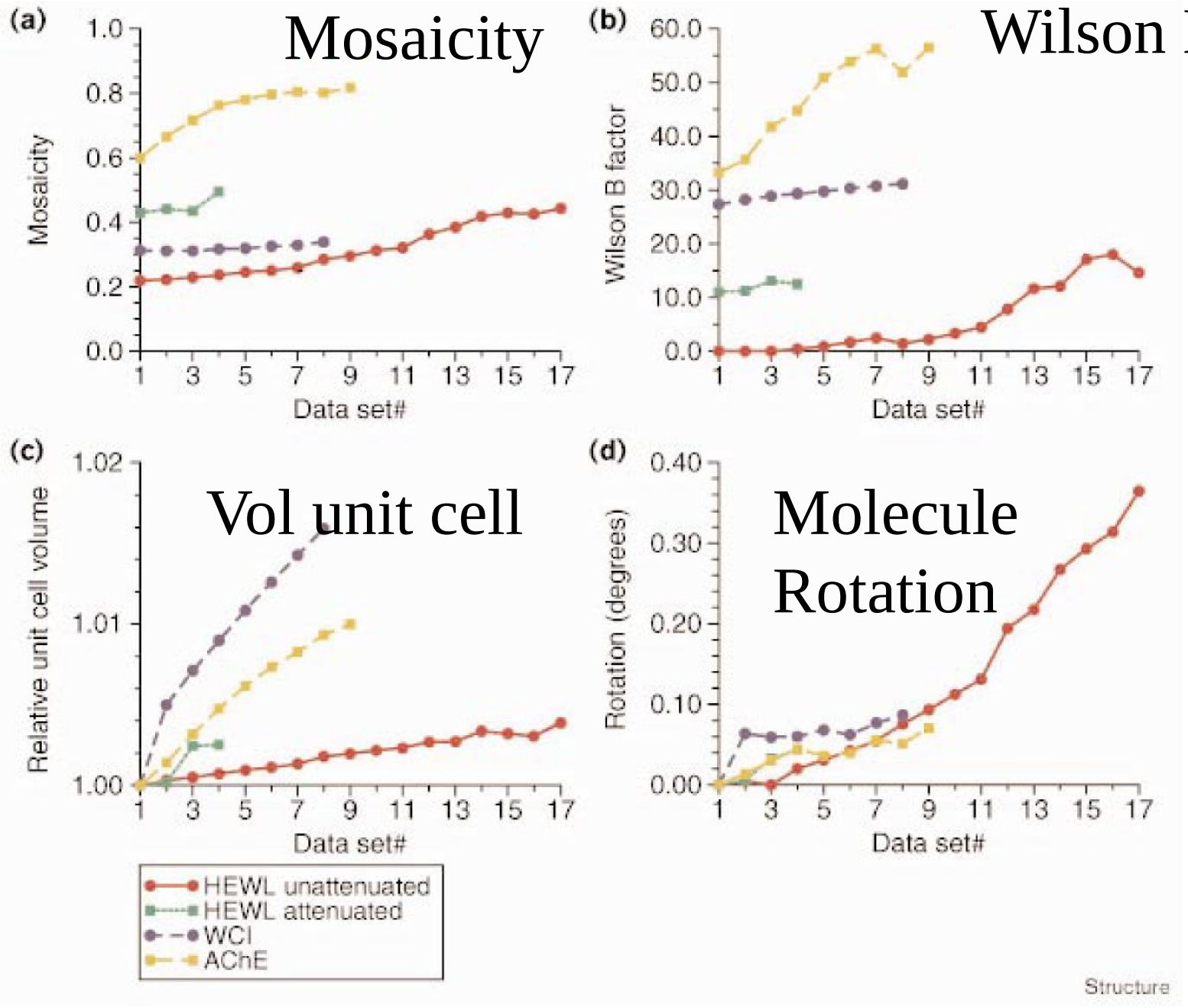
Intensity Decay at 100K

Normalised Intensity vs Dose: apoferritin



Unit cell volume increase





Ravelli and McSweeney, Structure (2000) 8, 315

Data Parameters affected by Radiation Damage

- $I/\sigma(I)$ or resolution limit ↓
- R_{merge} ↑
- Scaling B factors ↑
- Mosaicity ↑
- Unit Cell expansion a) function of dose ↑

  b) function of cryogen temperature ↑

Could this be an on-line damage metric?

[Ravelli and McSweeney, (2000) Structure]

No!

[Murray and Garman (2002), JSR, Ravelli et al (2002) JSR]

What global damage metric should we use and against what should we plot it?

- I_n/I_o
- Not I/sigma(I)
- Scaling B factors?
- An R_{meas} type measure?

To monitor the damage we need an *x*-axis!
Not time or image number...
Dose estimation

$$\text{Dose} = \frac{\text{energy absorbed}}{\text{unit mass}}$$

$$= \frac{\text{J}}{\text{kg}} = \text{Gy (gray)}$$

Fundamental metric against which to measure damage.

Takes care of the physics but NOT the chemistry.

RADDOSE-3D: www.raddo.se

MX experiment

1 MGy/s absorbed by a 100 μm cubed metal free crystal in a

100×100 μm^2 beam of

12.4 keV (1 Å) X-rays

flux: 10^{13} photons s^{-1}

3 Gy

MX at 100 K: 30 MGy experimental dose ‘limit’ reached in ~30 s:
4th generations sources <<1s,
XFELs: <80 fs

Intensity Decay at 100K

Normalised Intensity vs Dose:

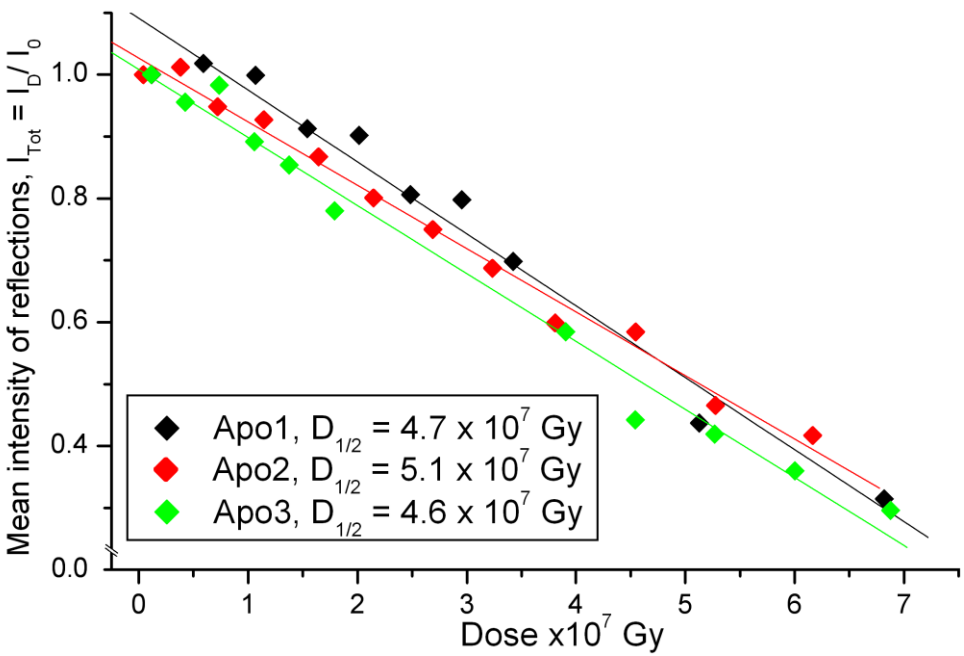
apoferritin

Coefficient of sensitivity α change in relative isotropic B factor:

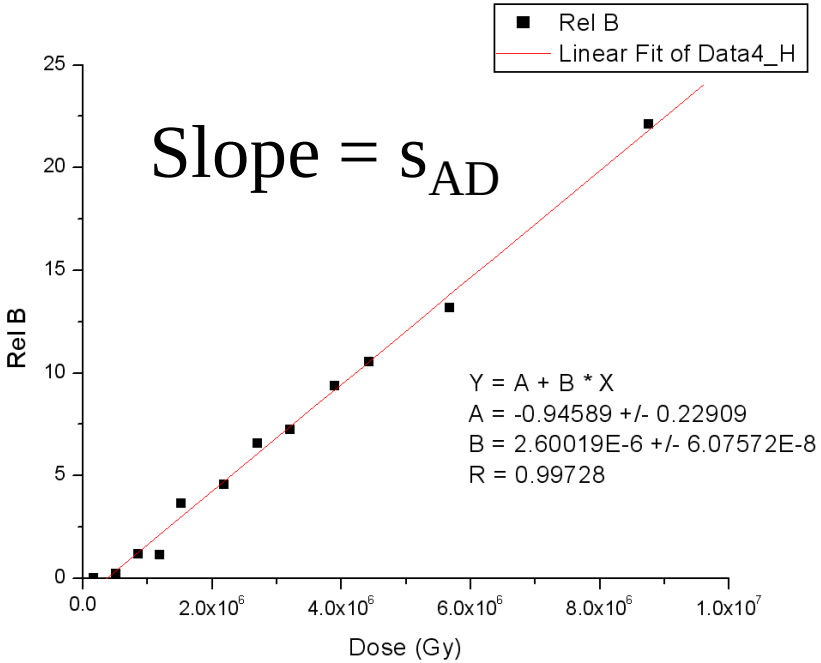
$$s_{AD} = \Delta B_{rel} / 8\pi^2 \Delta D$$

(e.g. HEWL@100 K = 0.012 $Å^2$/Gy)

[Owen et al 2006, PNAS]

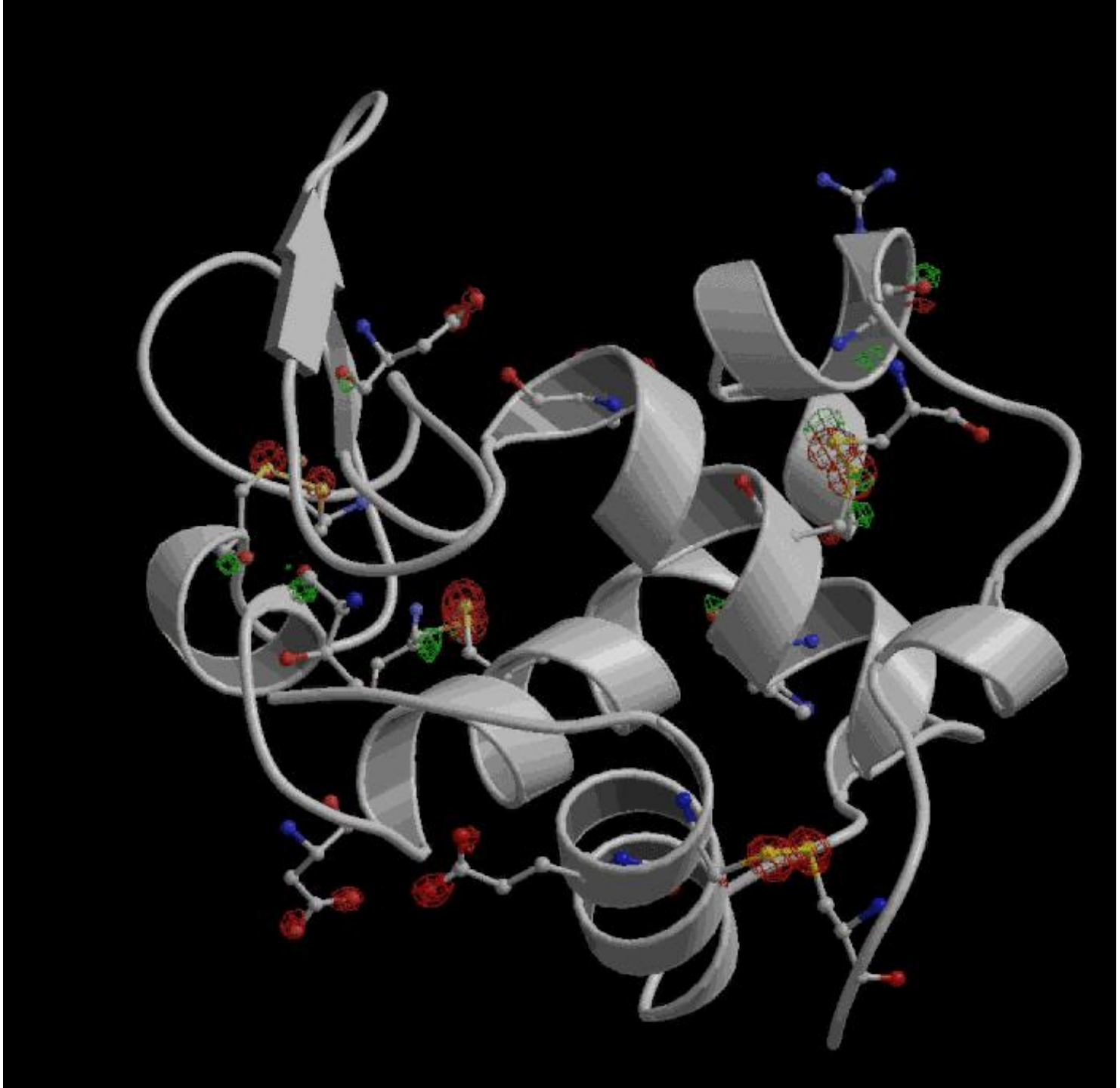


[Kmetko et al 2006, Acta D62]



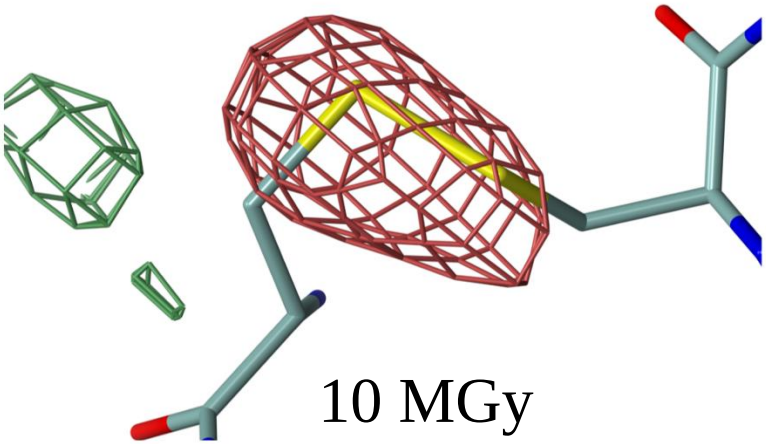
Global damage: summary

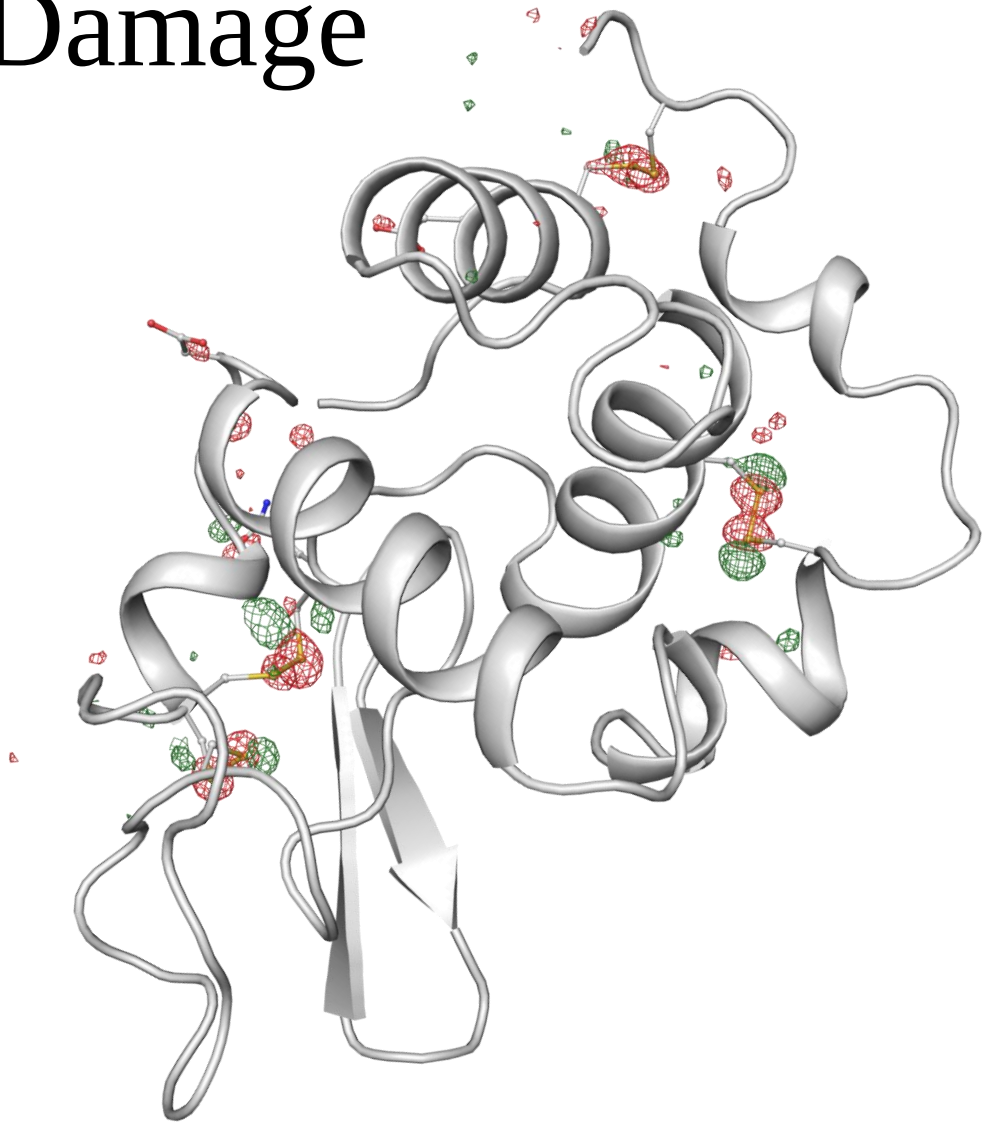
- Incomplete data/lost resolution/scaling ‘dip’
- Causes non-isomorphism within a dataset (unit cell grows)
- No significant ($< \times 2$) dose rate effect at 100 K at current flux densities (10^{15} ph/s/mm^2).
- No significant ($< \times 2$) temperature dependence below 100 K, but weak minimum at around 50 K.
- Damage to lattice due to hydrogen abstraction and then build up?
- Heating not significant at current flux densities.
- For a particular system is predictable/can be modelled (using a sacrificial crystal)



Specific Damage

- Specific damage occurs in a predictable hierarchy in proteins
- Reduction of metallocentres
- Breakage of disulphide bonds
- Asp and Glu decarboxylation





Difference map Fo_4-Fo_1

Weik et al. 2000, Burmeister 2000
Ravelli and McSweeney 2000,

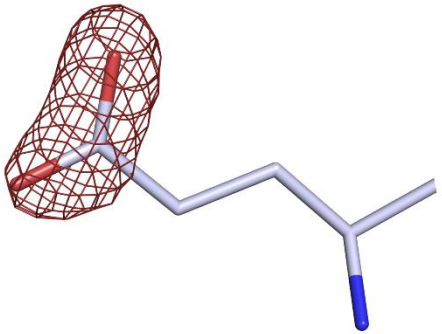
Specific Damage

- Specific damage occurs in a predictable hierarchy in proteins
- Reduction of metallocentres
- Breakage of disulphide bonds

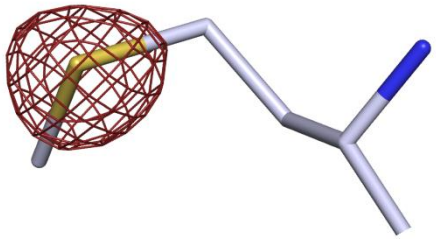
- Decarboxylation of ASP and GLU residues
- Cleavage of S—C bond in MET
- Rupture of covalent bonds to heavier atoms:

  C-Br, C-I, S-Hg

- **Note** that if this were due to primary damage alone, damage would be in order of absorption cross sections of atoms, which it is not.



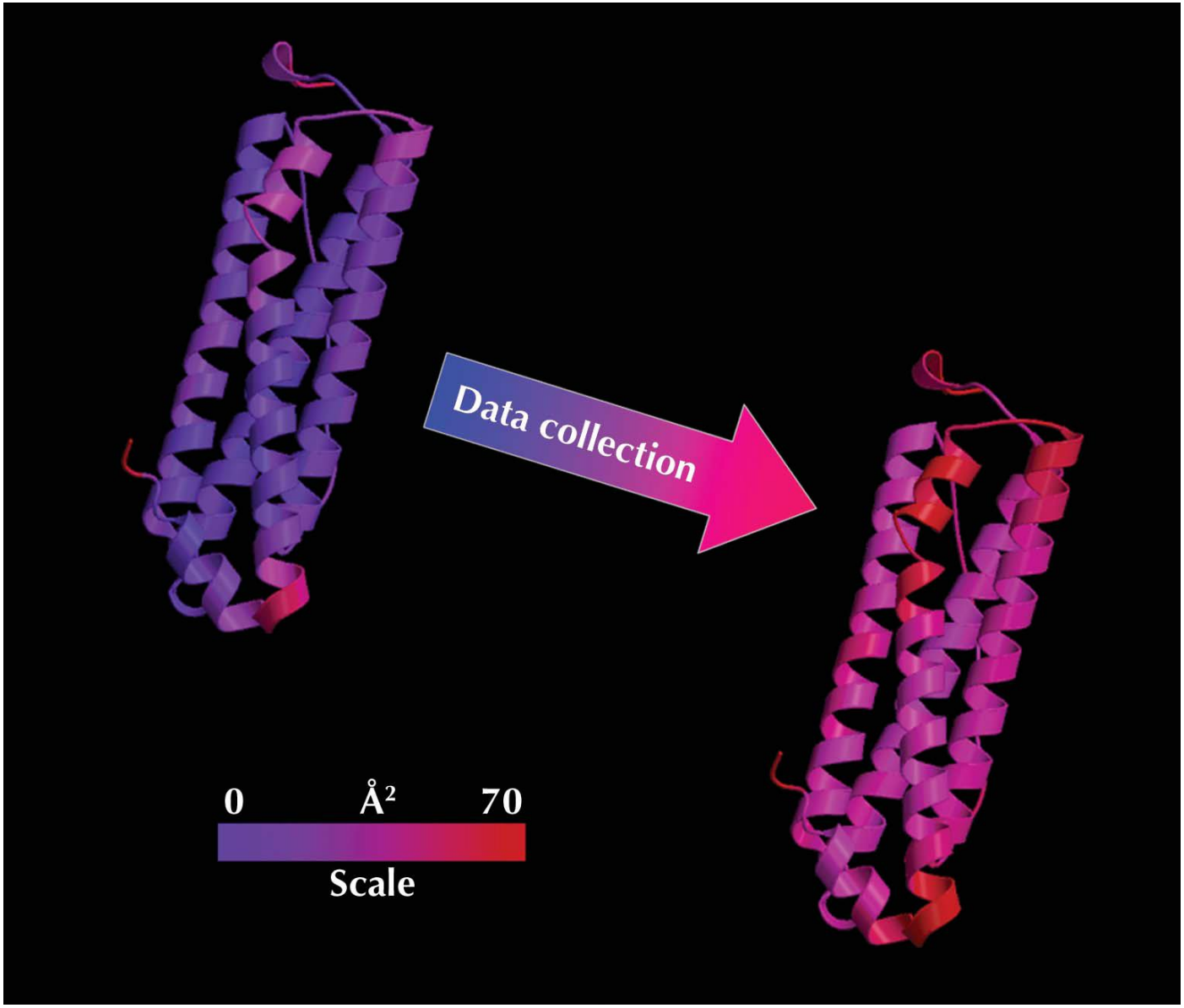
GLU ±4σ–level difference map: Red =negative density



MET ±4σ–level difference map: Red = negative density

Bury et al., JSR 2015

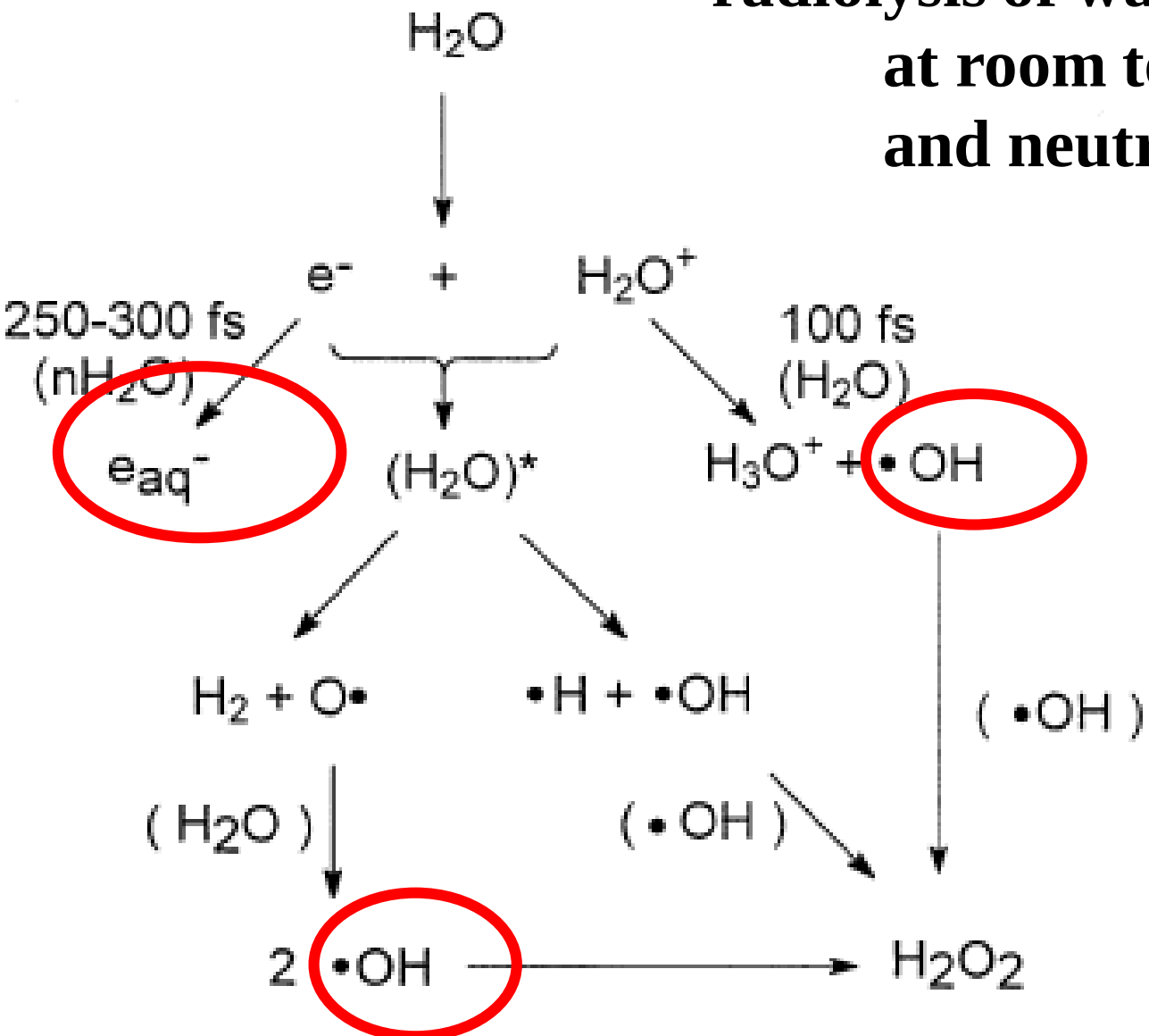
ALSO:
Atomic B-factors increase:



Damage: the Radiation Chemistry

1) INDIRECT RADIATION DAMAGE :

radiolysis of water at room temperature and neutral pH:



OH thought not to be mobile in glasses below 110K

(Owen et al Acta D 2012)

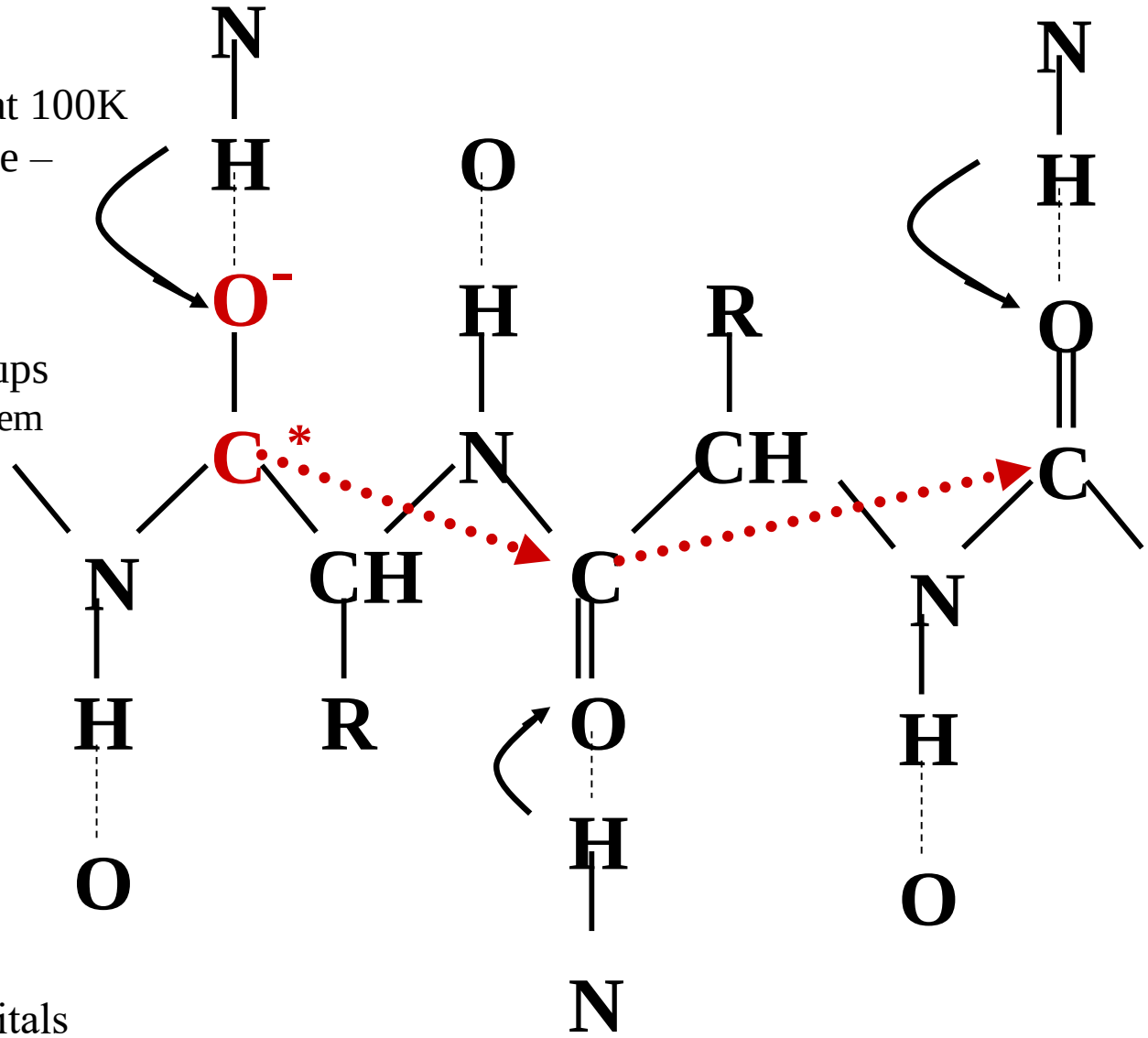
Hiroki, A. Pimblott, S. M. LaVerne, J. A. (2002) *J Phys Chem A* **106**, 9352-9358

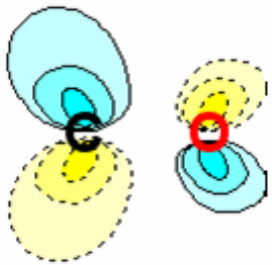
2) DIRECT RADIATION DAMAGE. Protein Redox

a) electron migration and trapping.

Excess electron migrates even at 100K (q.m.tunnelling) along backbone – trapped at a unique C=O

ESR: electrons mobile at 77K
Go to most electron affinic groups
M. Symons 1995, Radiat. Phys. Chem 45, 837-845.





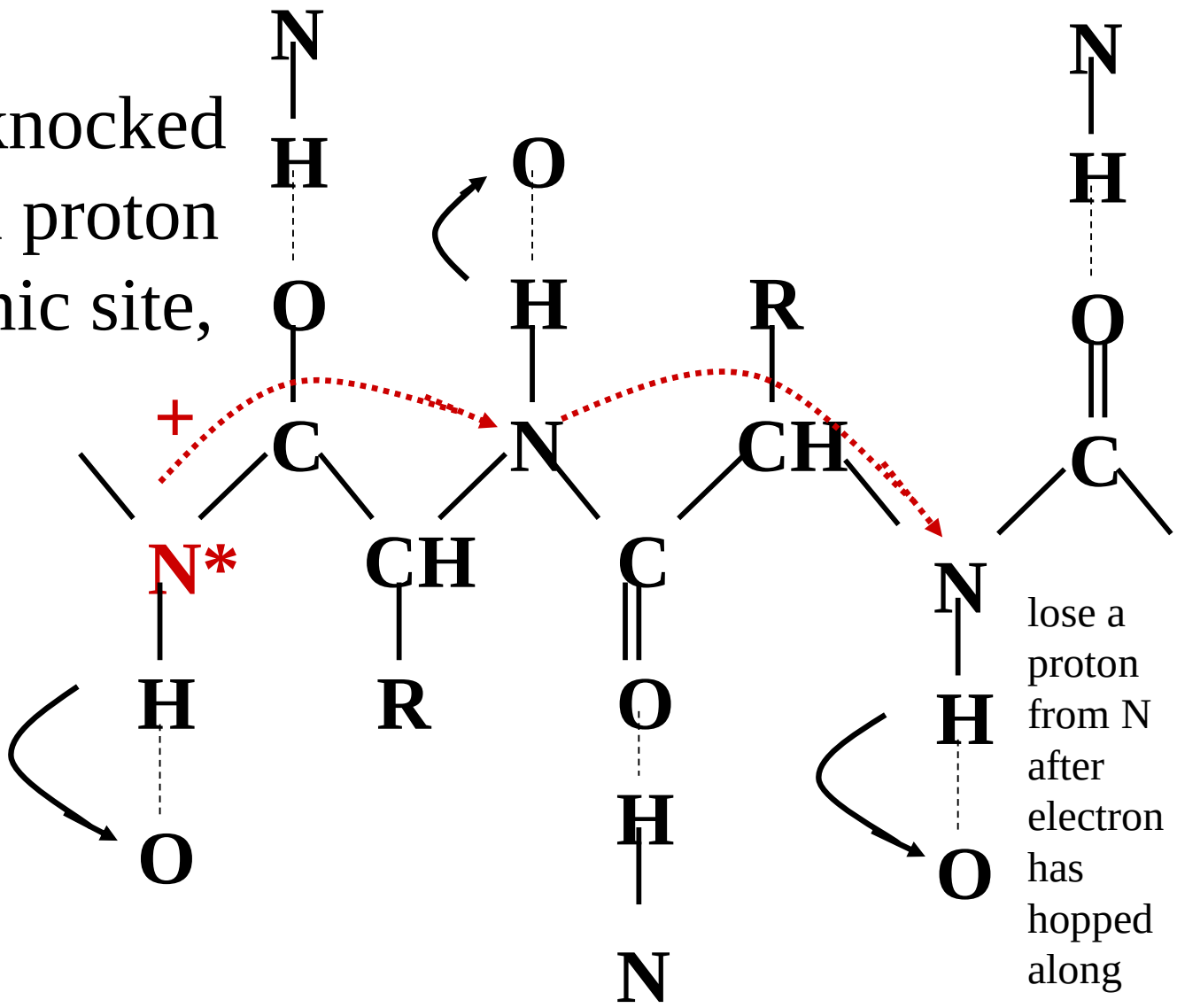
π* orbitals

2) DIRECT RADIATION DAMAGE. Protein Redox-

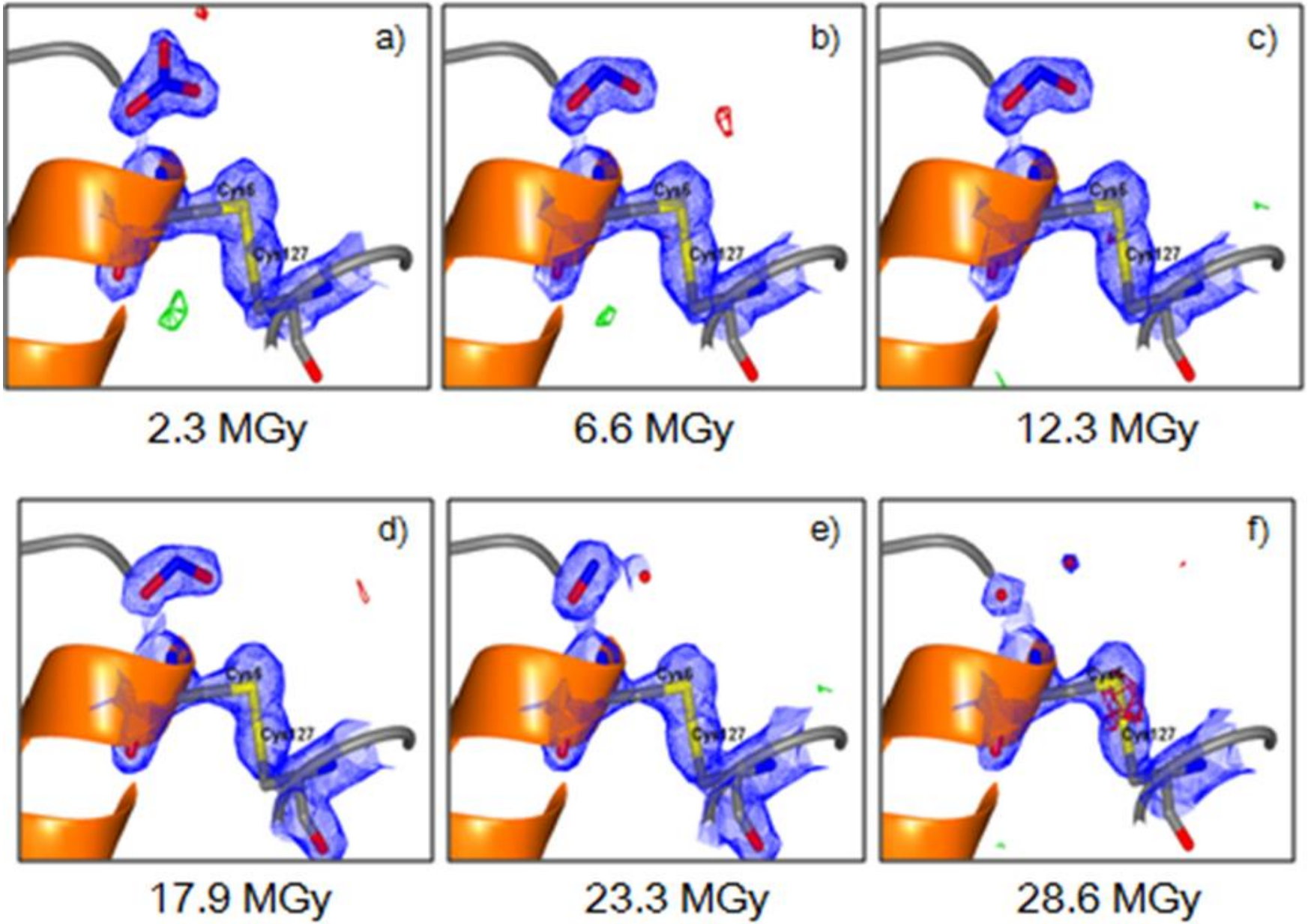
b) proton hole migration.

Electron gets knocked off, then lose a proton from the cationic site, get a radical.

lose an electron from N



Radiation Chemistry in action: Nitrate scavenger



De la Mora, Carmichael, Garman *JSR* (2011) 18, 313

Specific Damage: summary.

- Can compromise biologically relevant observations (e.g. damage enzymatically important glutamates).
- Metallo-enzymes are reduced by X-ray beam.
- Perhaps weakly dose rate dependent ($< \times 2$)
- Perhaps weakly wavelength dependent ($< \times 2$)
- Weakly temperature dependent (varying results) ($< \times 2$)
- Can be reduced with certain scavengers: but very conflicting results (mainly $< \times 2$, benzoquinone RT $\times 9$)
- We DON'T understand pecking order of damage within an amino acid group pH? Solvent accessibility? Neighbouring amino acids?

Radiation damage:
The Plan:

- What are the symptoms?
- **What is it?**
- Why do we care? Effect on MAD/SAD.
- How do we calculate the Dose?
- What do we know/would like to know?

PHYSICS of the interaction of X-rays with crystals.

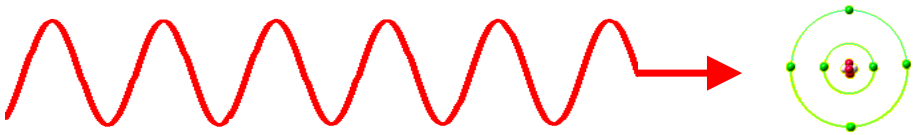
A) Diffraction

B) Absorption = Energy loss

N.B. > 90% of the beam does not interact at all,
but goes straight through.

A) Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering

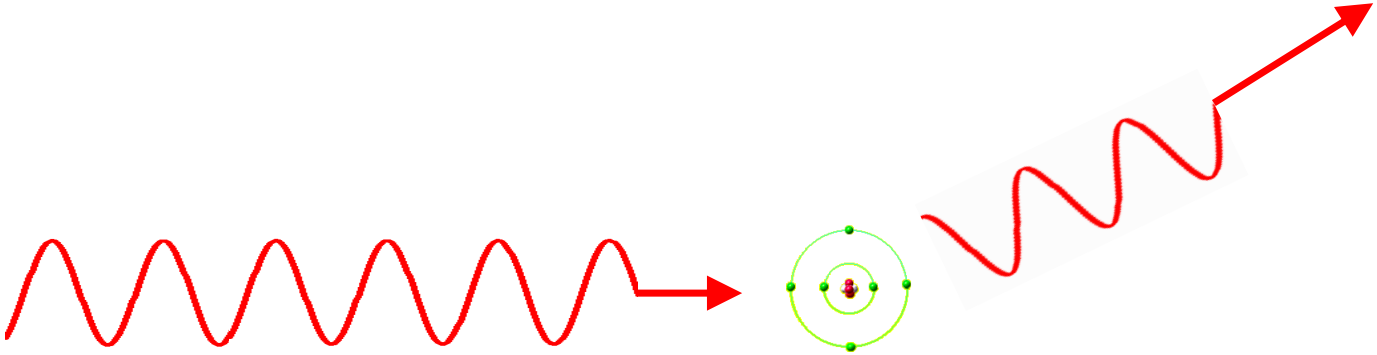


[8% at 1Å]

ELASTIC - no energy loss.

Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering



[8% at 1Å]

ELASTIC - no energy loss.

Primary X-ray interaction processes with crystal and solvent.

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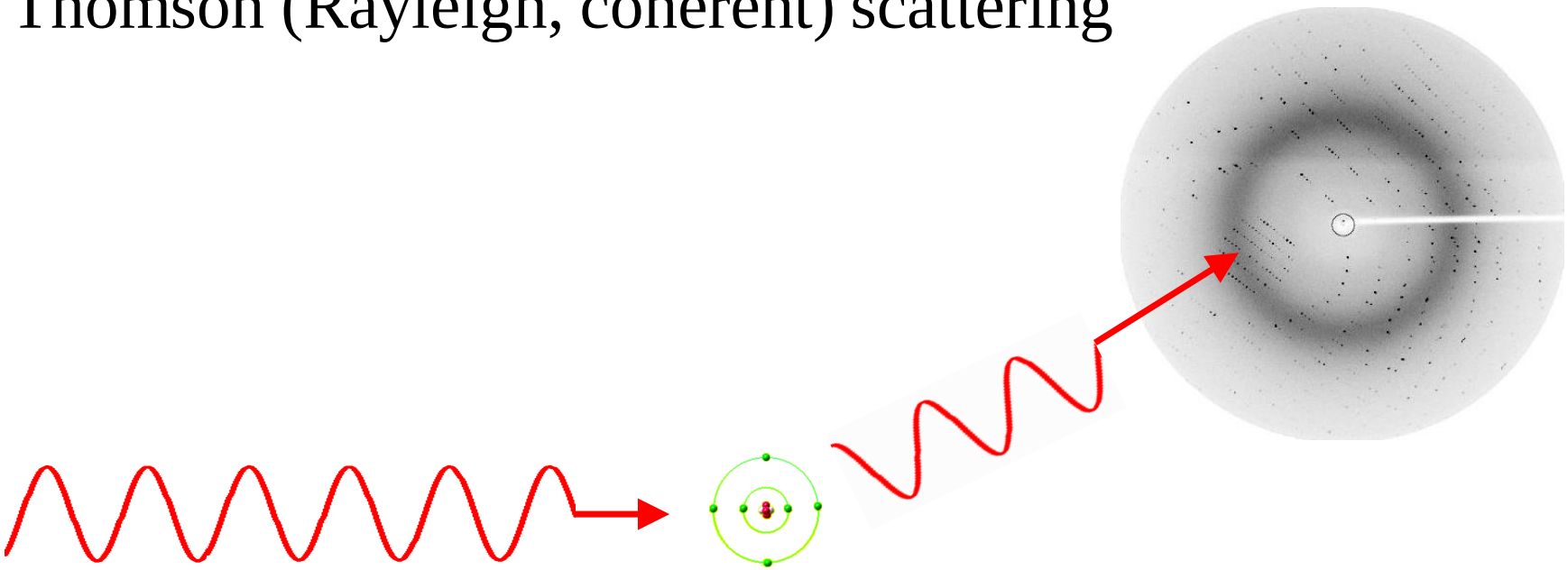


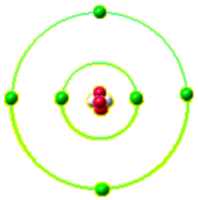
ELASTIC - no energy loss.

Coherent – adds vectorially and gives diffraction pattern.

Small proportion of total scattering: 8% at 1Å

BUT IT IS THE BIT WE WANT!!

Compton (incoherent) scattering



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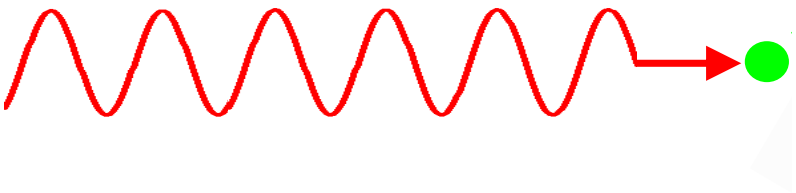
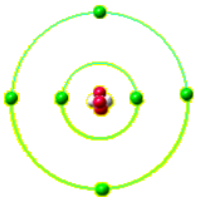


X-ray transfers some energy to atomic electron and thus has lower energy (higher wavelength).

Compton (incoherent) scattering



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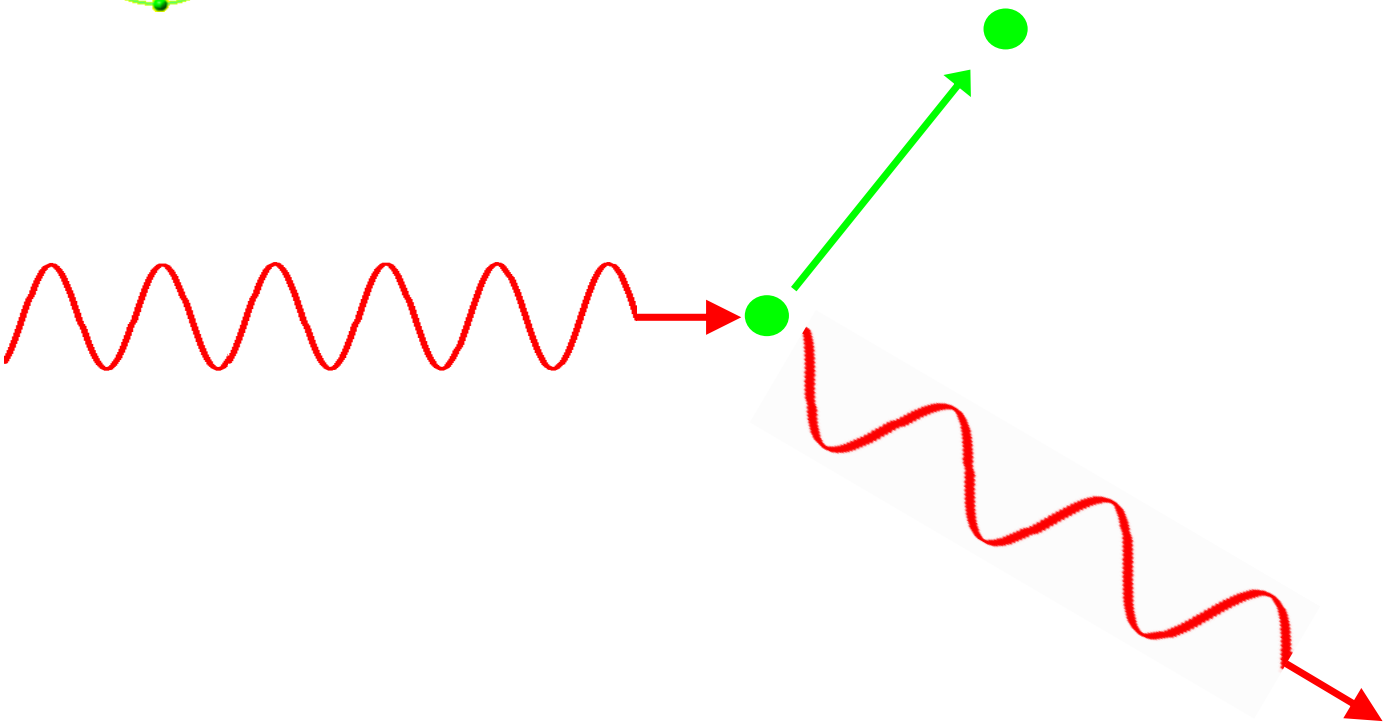


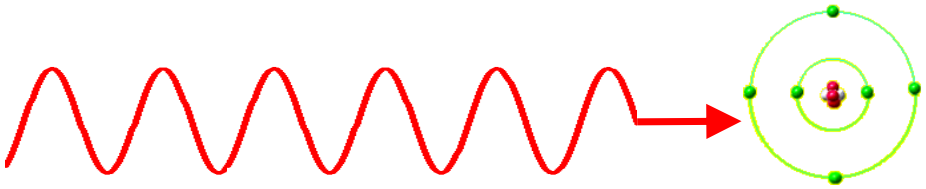
X-ray transfers some energy to atomic electron and thus has lower energy (higher wavelength).

Incoherent – part of X-ray background in images.

Also a small proportion of total scattering: 8% at 1Å

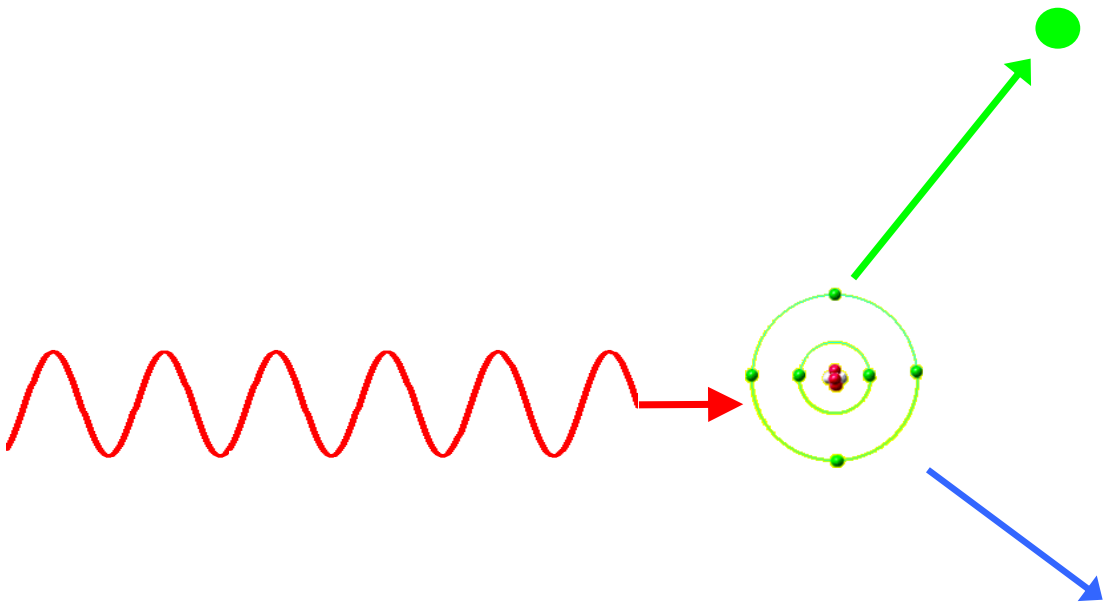
Photoelectric Absorption

84% at 1Å



INELASTIC.

Photoelectric Absorption



84% at 1Å

INELASTIC.

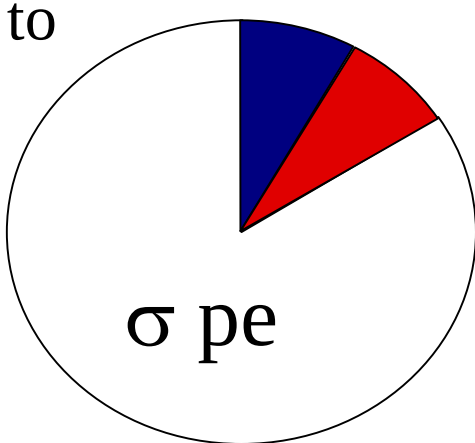
X-ray transfers all its energy to an atomic electron, which is then ejected.

Each 12 keV primary photoelectron can give rise to up to 500 ionisation events.

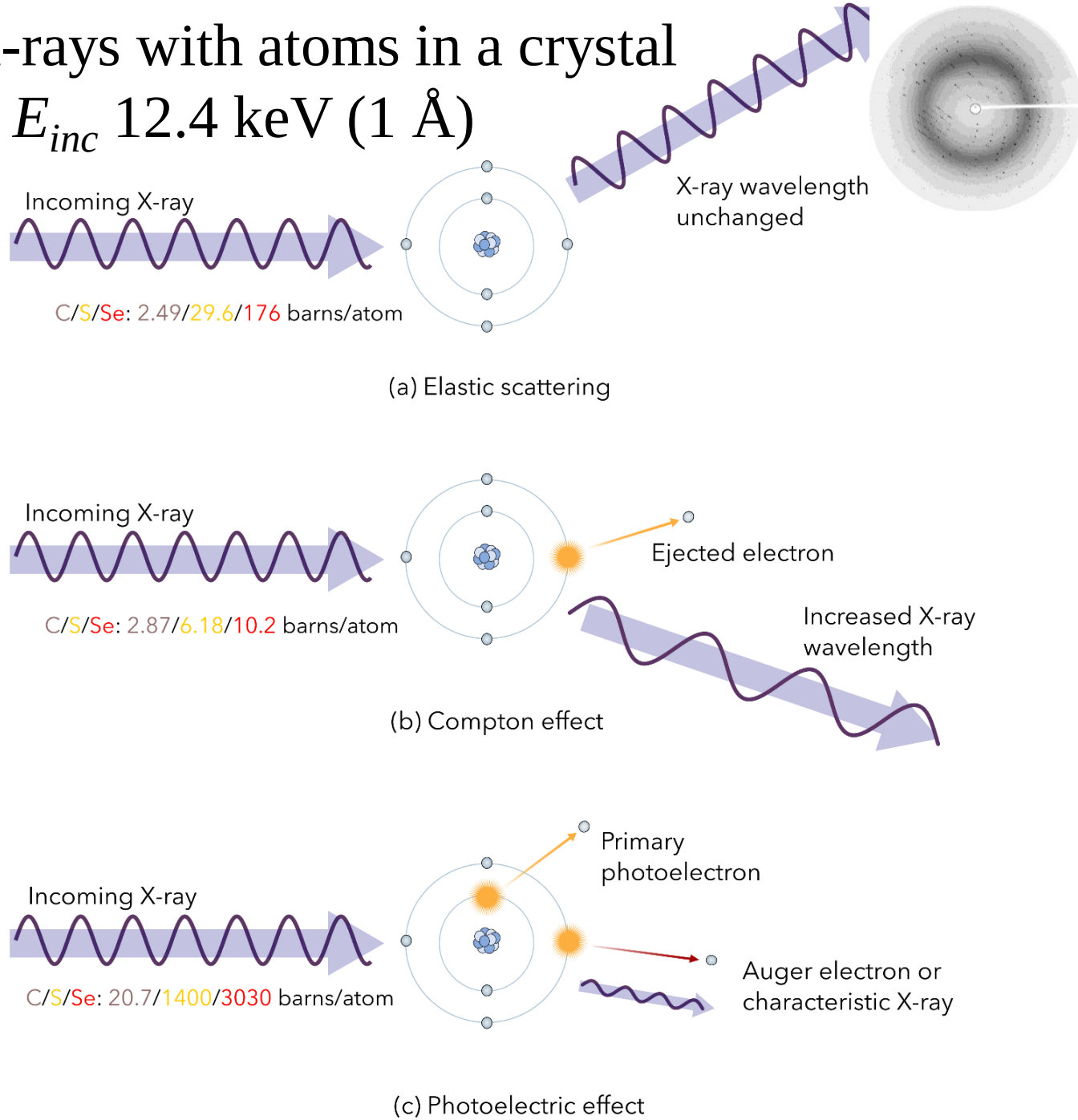
Atom can then emit a characteristic X-ray or an Auger electron to return to its ground state.

$$\sigma tot = \sigma\ pe + \sigma\ inc + \sigma\ coh$$

$$84\% + 8\% + 8\%$$



Interactions of X-rays with atoms in a crystal
Cross sections at E_{inc} 12.4 keV (1 Å)



Photoelectric Cross Sections (barns/atom) at 13.1 keV

[1 barn=$10^{-28}m^2$]

A few heavy atoms can make a big difference.

.

H

[Ravelli *et al.*, JSR,(2005) 12]

Photoelectric Cross Sections (barns/atom) at 13.1 keV

[1 barn=10^{-28}m^2]

A few heavy atoms can make a big difference.

[Ravelli *et al.*, JSR,(2005) 12]

Photoelectric Cross Sections (barns/atom) at 13.1 keV

[1 barn=10^{-28}m^2]

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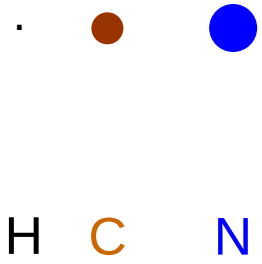

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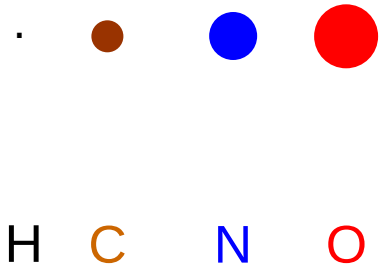

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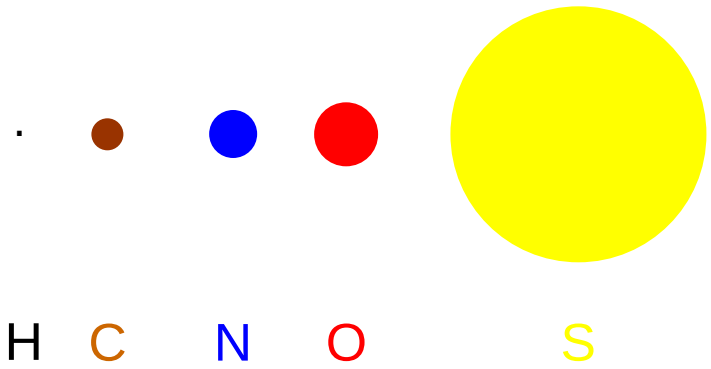

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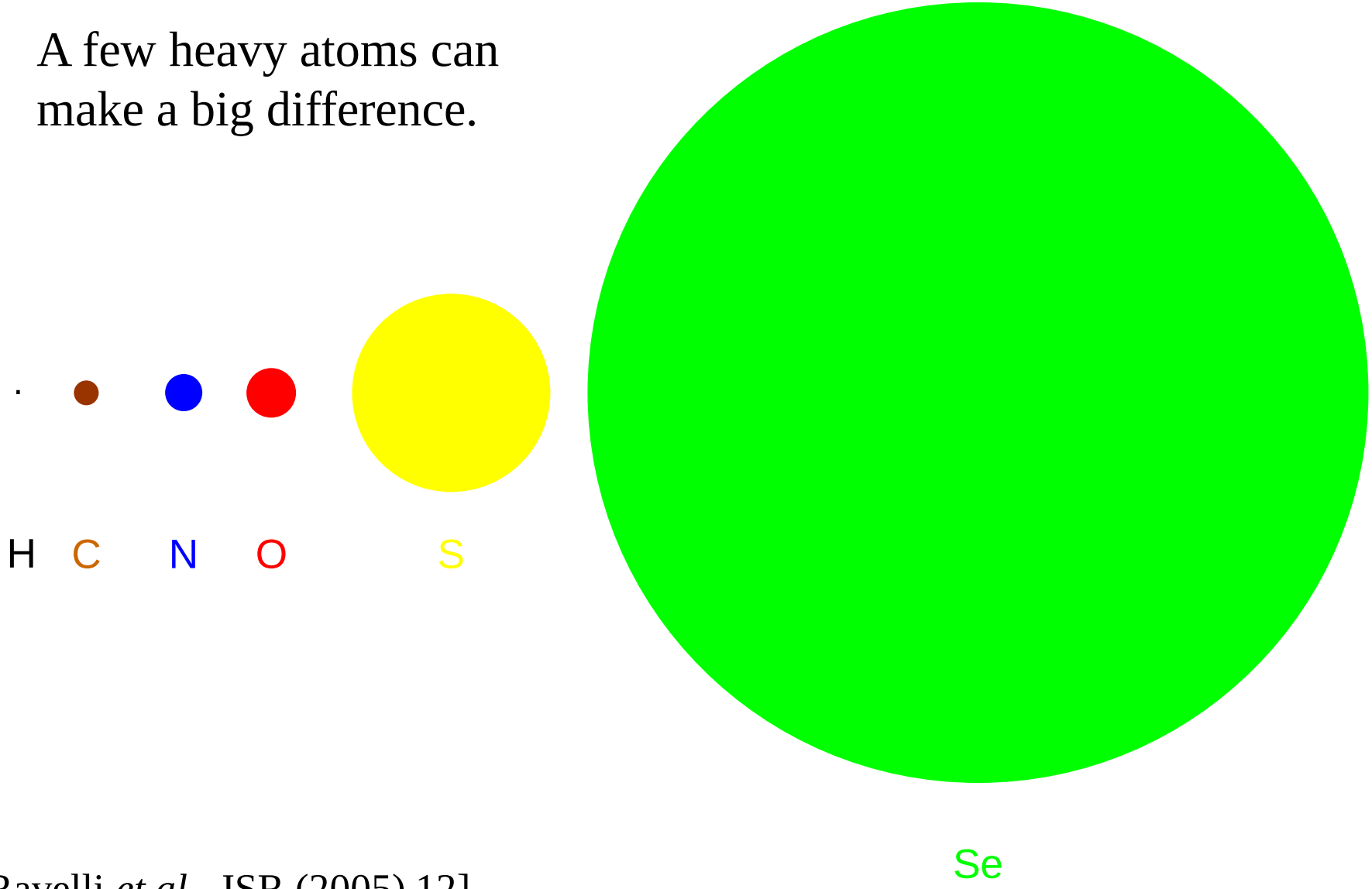
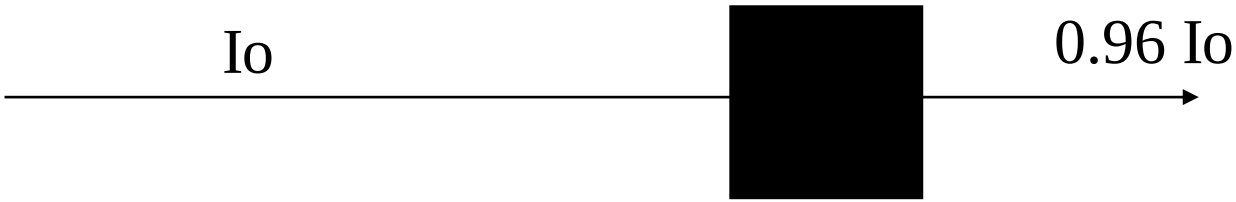


[Ravelli *et al.*, JSR,(2005) 12]

Beam absorption (λ=1Å) by a protein crystal

Native HEWL 100 µm thick

Platinum derivatised (1/molecule)
HEWL 100 µm thick



N.B. INCIDENT FLUX is the SAME but the absorbed dose is DOUBLE

A few heavy atoms in the solvent can make a BIG difference to the absorption cross section and this the dose rate for the SAME flux.

e.g. Cacodylate buffer (arsenic, mass 75 cf selenium = 79)

BACK SOAKING to REMOVE

Non-specifically bound heavy atoms

e.g. a brominated DNA-protein complex will radiation damage much faster than a native crystal, and will de-brominate during data collection [Ennfar et al, Acta Cryst D (2002) 1263-1268].

Radiation damage:
The Plan:

- What are the symptoms?
- What is it?
- **Why do we care? Effect on MAD/SAD.**
- How do we calculate the Dose?
- What do we know/would like to know?

Effect on MAD/SAD phasing methods.

- Failure of structure determination

(Multi-wavelength anomalous dispersion MAD, SAD)

due to creeping non-isomorphism –

a) cell expansion and

b) movement of molecule in unit cell

c) structural changes DURING experiment.

i.e. **MAD/SAD phasing** signals (<5%) washed out completely.

Non-isomorphism: DISASTER!

Crick and Magdoff (1956) showed that for a 0.5% change in all three unit cell dimensions of 100Å, the intensity would change by

15% at 3Å

for general reflections

[Crick and Magdoff (1956) Acta Cryst **9**, 901-908]

i.e. **MAD/SAD phasing** signals (<5%) washed out completely.

Radiation damage:
The Plan:

- What are the symptoms?
- Why do we care? Effect on MAD/SAD.
- What is it?
- **How do we calculate the Dose?**
- What do we know/would like to know?

DOSE Postulate (Henderson 1990):

- There is a MAXIMUM dose

(Energy absorbed/unit mass: Joules/kg = Gy)

which protein crystals can tolerate which depends only on the PHYSICS of the situation.

- Crystal might not reach that limit due to chemical factors, but it is unlikely to last BEYOND the limit.

- Need to be able to calculate the DOSE:

RADDOSE

V1: Murray, Garman & Ravelli (2004) JAPC, 37, 513-522
V2: Paithankar, Owen & Garman, (2009) JSR 16, 152-162
V3: Paithankar & Garman (2010), Acta D 66, 381-388

Calculated radiation dose limit for biological specimens at 77K from analogy with electron microscopy.

[Henderson (1990) Proc. R. Soc. Lond. B **241**, 6-8.]

- For 100 keV electrons, diffraction from protein crystals at 77 K fades to half the intensity with 5 electrons/$Å^2$

- This corresponds to a dose of: 5×10^7 Grays

[say $\mathbf{2 \times 10^7}$ Grays in first part of depth-dose curve (~50μm)] ***

(1 Gray = 1Joule kg^{-1})

5 electrons/$Å^3$ ***

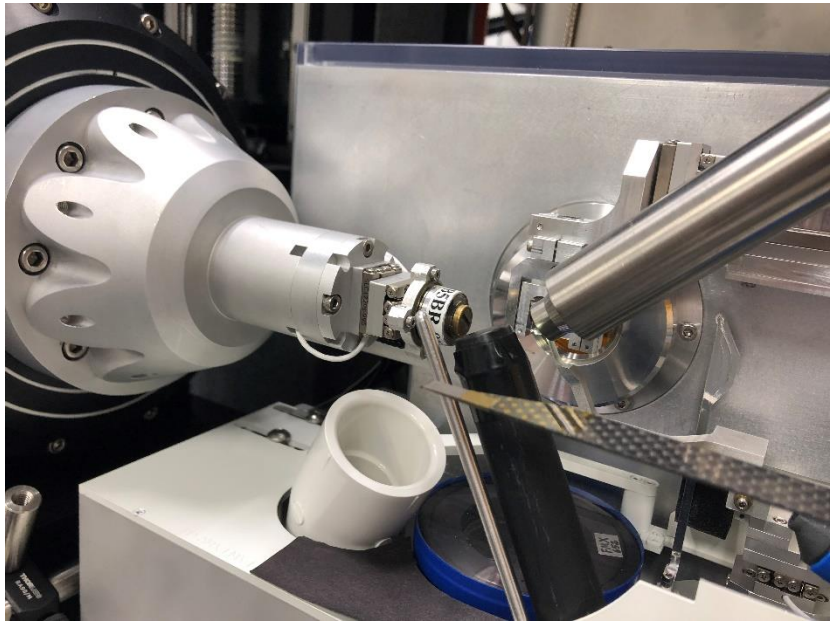
100 keV e^- →

Absorbed energy to take diffracted Io → Io/2

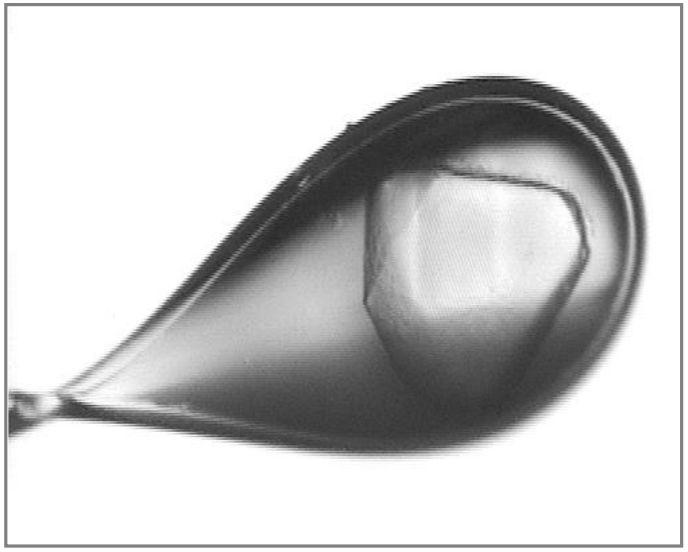
Make the dose calculation convenient for MX (include solvent contribution in mM and heavy atoms explicitly)

To find the energy deposited per unit mass in the crystal, need to characterise two things:

The beam

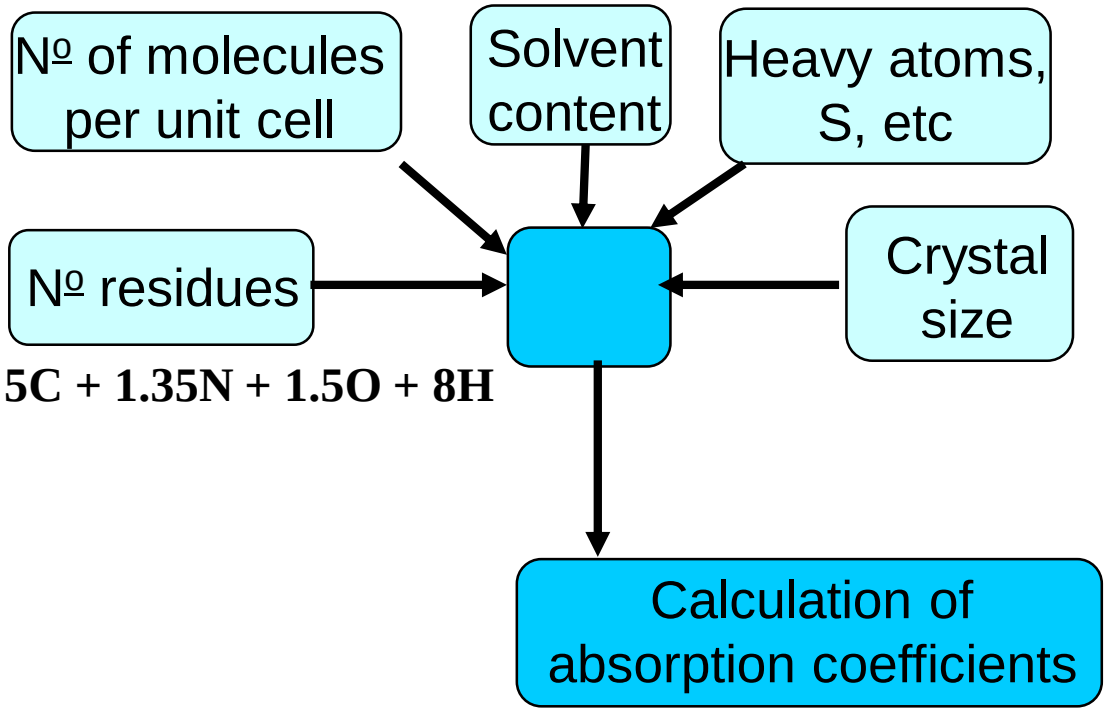


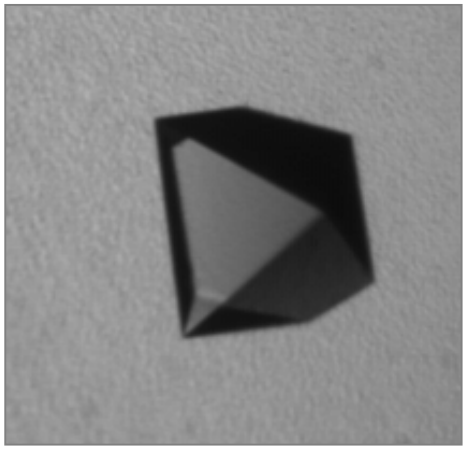
The crystal

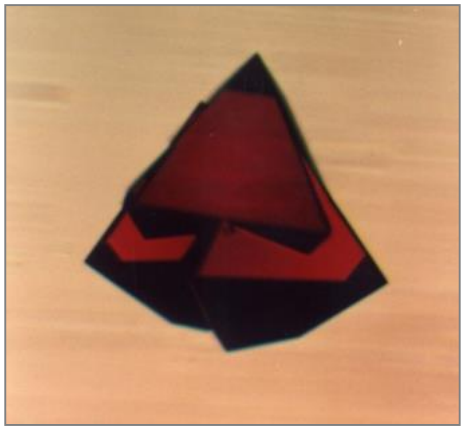


Calculating Dose
(*RADDOSE-3D*)

Crystal Characteristics





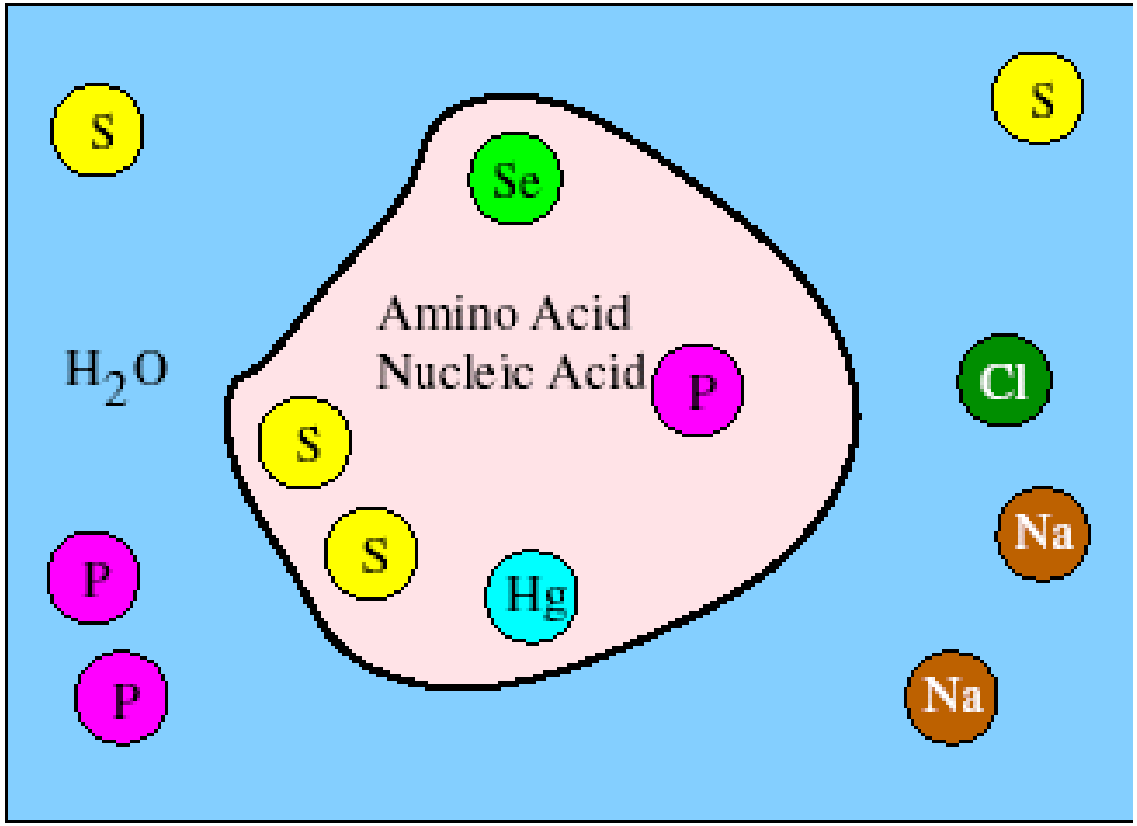


200 μm

absorption coefficients

e.g. apoferritin: 0.406mm^{-1}

holoferritin: 1.133mm^{-1}

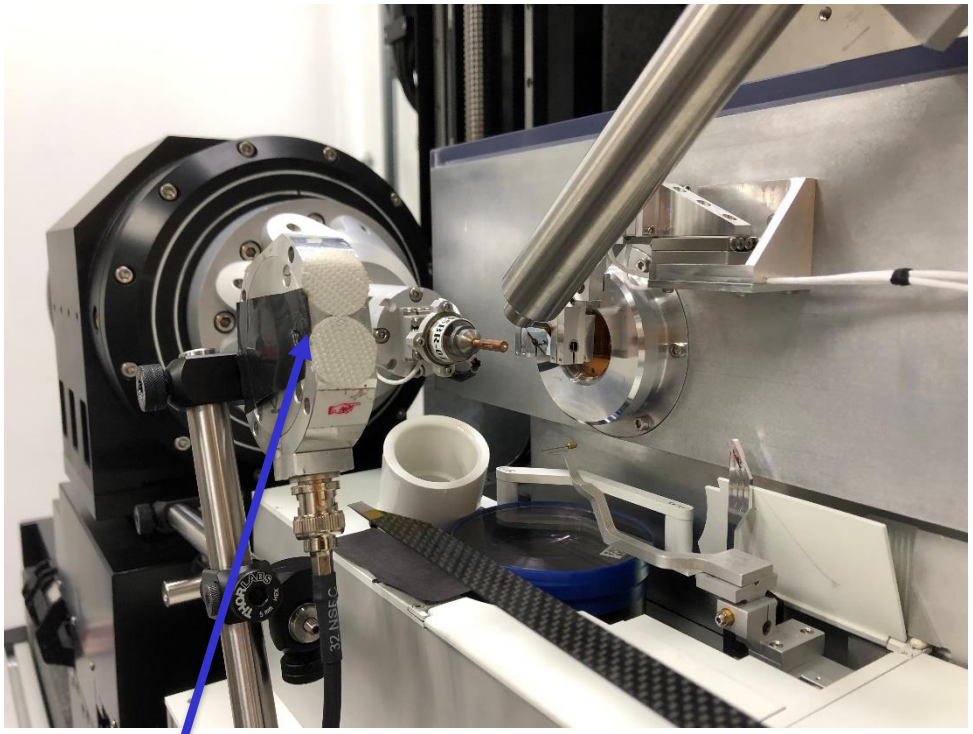


Number of Amino Acids

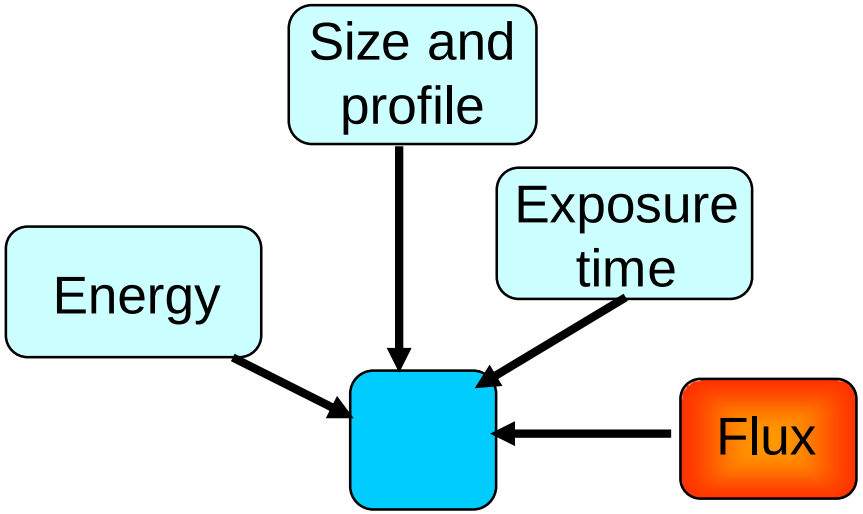
‘HA’ atoms per monomer, *e.g.* S, Se, Hg

Solvent - concentrations of components, *e.g.* Na^+, Cl^-

Calculating Dose
(*RADDOSE-3D*)
Beam Characteristics

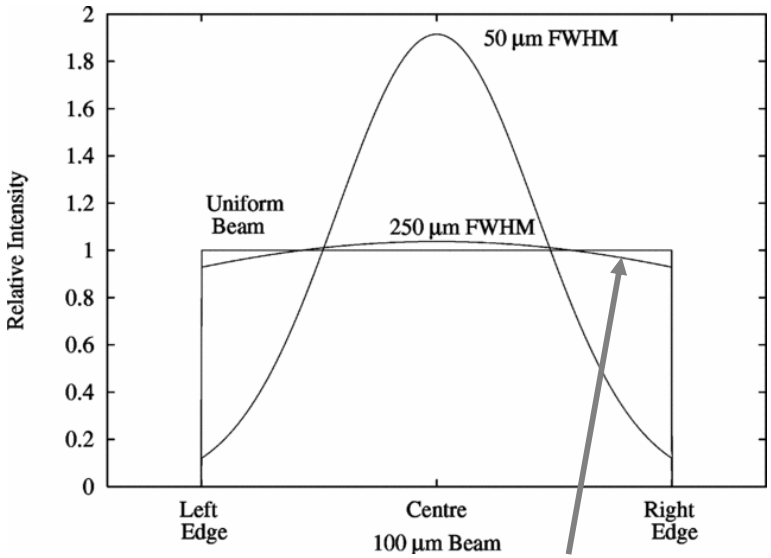


PIN diode



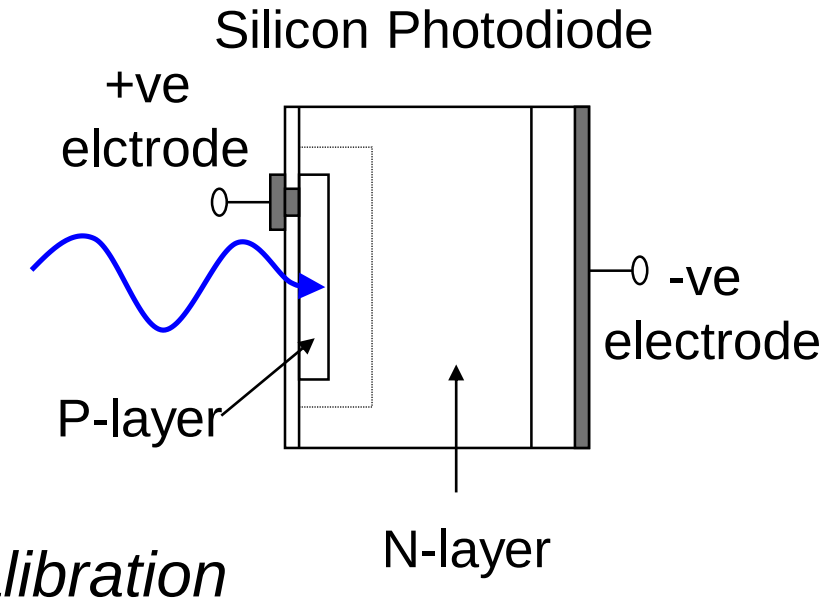
Beam Characteristics

Beam profile

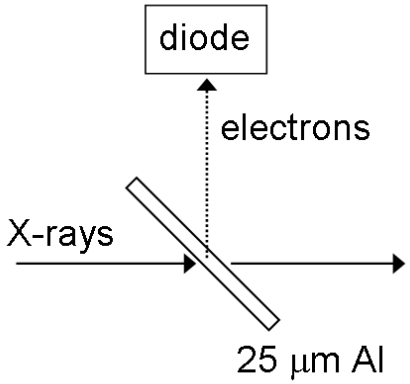


e.g. ID14-4, ESRF
P14, PETRA III

Flux: photons per second



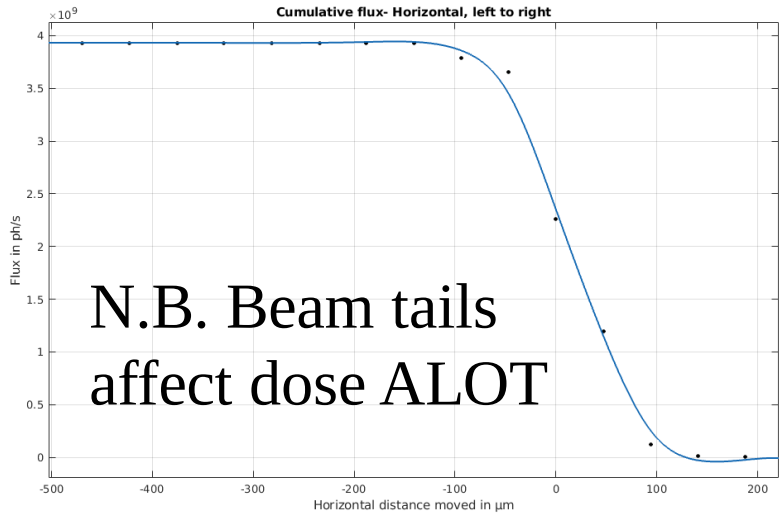
Calibration



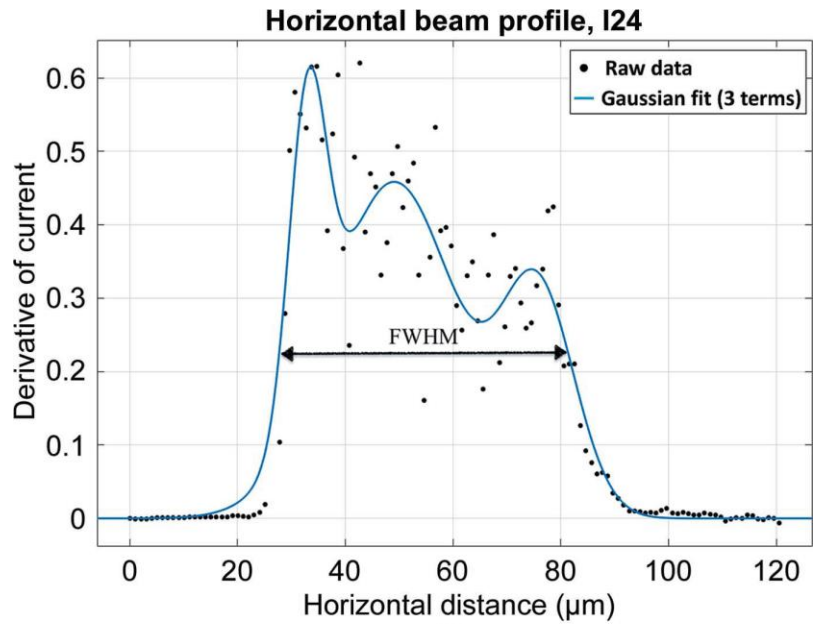
Photon Wavelength/Energy

Differential irradiation leads to differential damage: summation of states

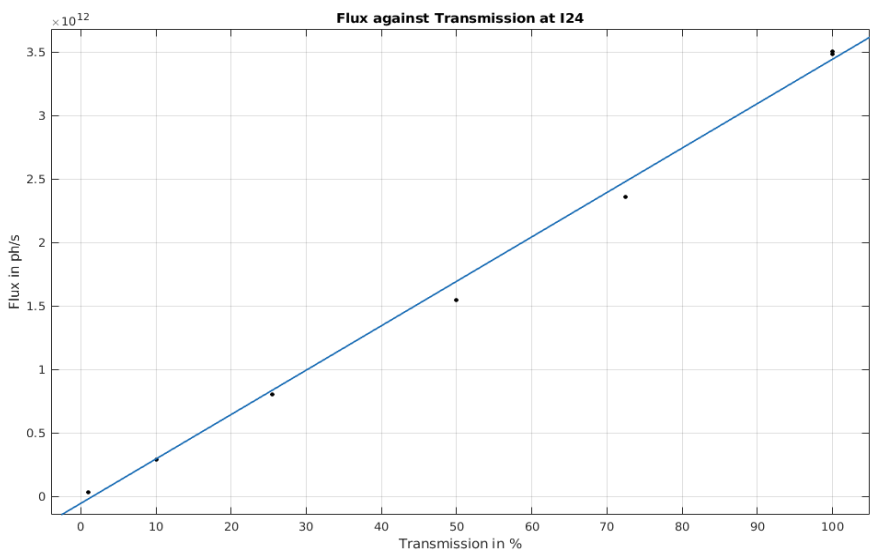
Beam profiles: measured with a wire or knife edge scan, then take derivative

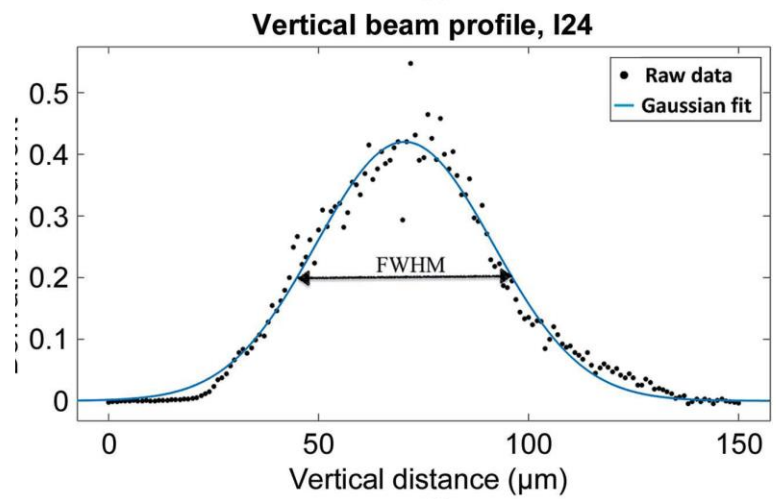


N.B. Beam tails affect dose ALOT



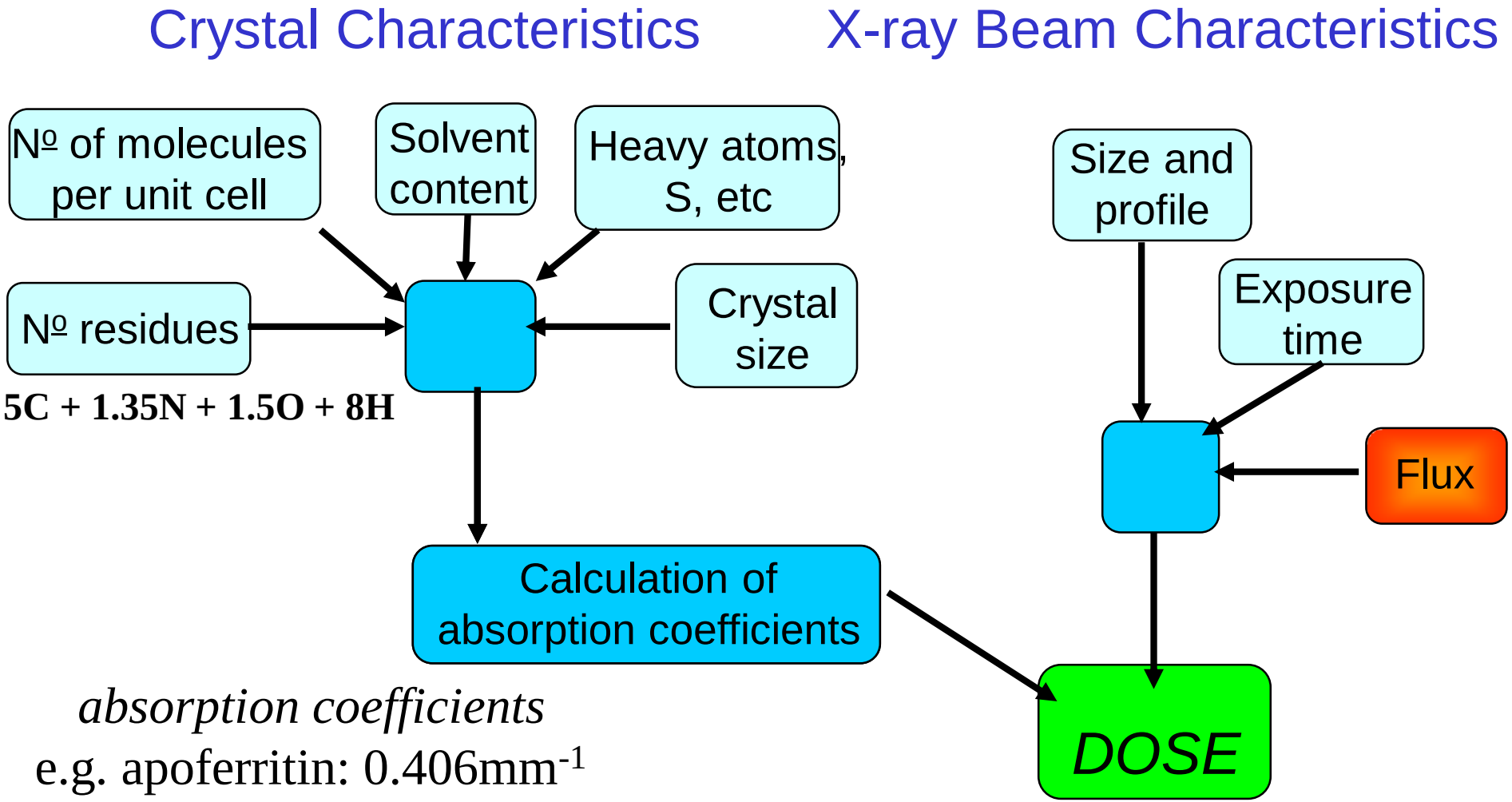
(*a*)





(*b*)

Calculating Dose
(*RADDOSE*)



absorption coefficients

e.g. apoferritin: 0.406mm^{-1}

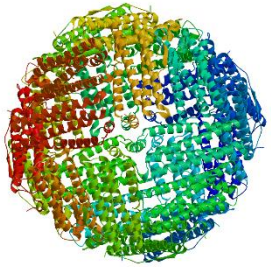
holoferritin: 1.133mm^{-1}

V1: Murray, Garman & Ravelli (2004) JAPC
V2: Paithankar, Owen & Garman, (2009) JSR
V3: Paithankar & Garman (2010), Acta D

MX experimental dose limit (@ 2Å) measurement: Ferritin

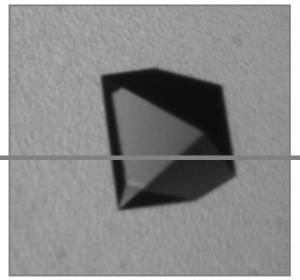
N.B. INCIDENT FLUX is the SAME but the absorbed dose is DOUBLE

The heavy atom ($z \geq 16$) content of a crystal is not crystallographically defined, but we need it.



Apoferritin

Io

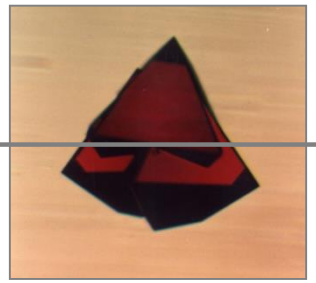


0.98 Io

absorption coefficient
$0.406 mm^{-1}$

Holoferritin

Io



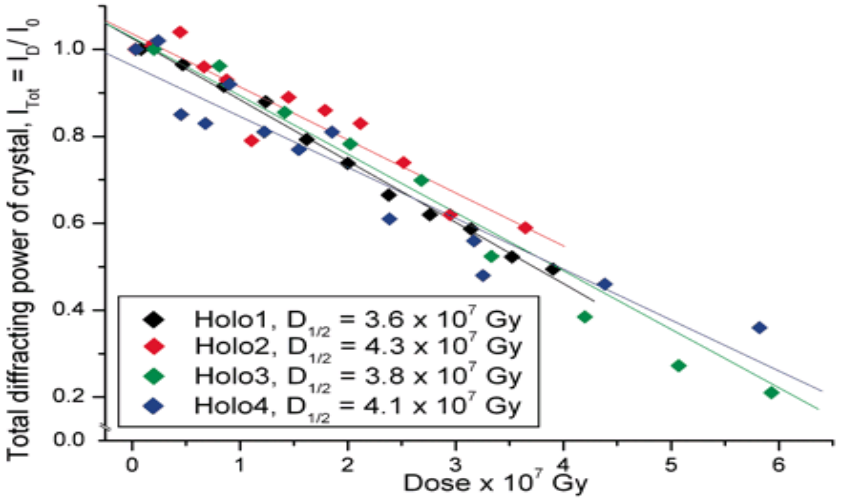
0.96 Io

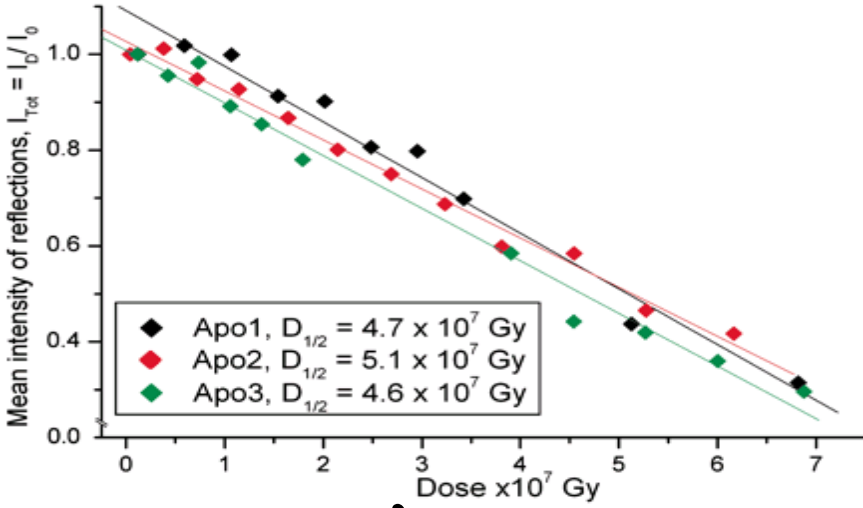
absorption coefficient
$1.133 mm^{-1}$

λ=1Å
100 μm thick xtal

Dose Limit Quantification at 100 K

- Holoferritin & Apoferritin as model: absorption coefficients differ by factor of 2
- Intensity: ~linear dependence on dose
- $D_{1/2}$ is dose to $I_0/2$
- $D_{1/2} = 4.3\ (\pm\ 0.4) \times 10^7$ Gy
  $= 43$ MGy
- Henderson limit, $D_{1/2} = 20$ MGy
- Howells et al (2005): resolution dependent limit of 10 MGy/ Å





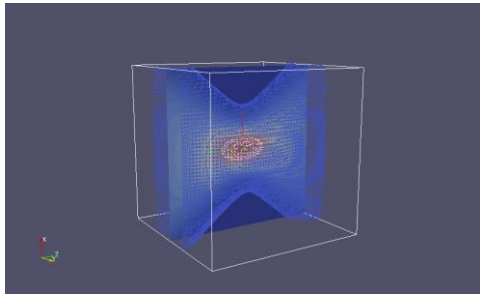
Suggested limit to retain biological `fidelity'

$$D_{0.7} = 3.0 \times 10^7 \text{ Gy} = \mathbf{30\ MGy}$$

Owen, Rudiño-Piñera, Garman PNAS (2006)

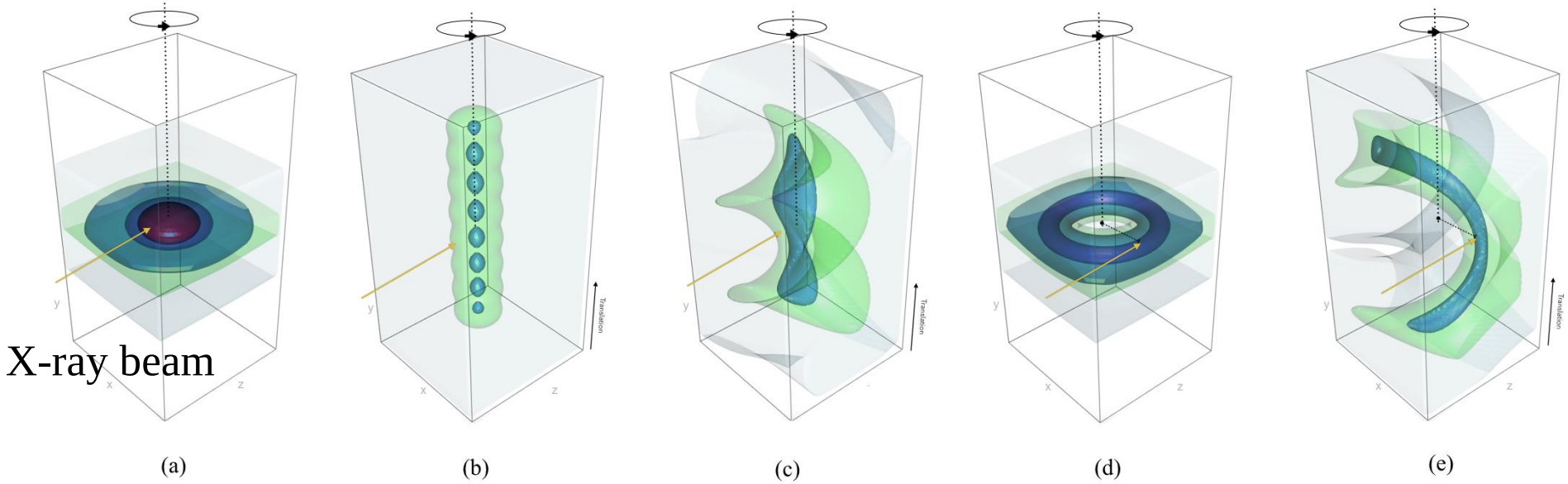
RADDOSE-3D

- Full 3-D simulation of dose absorption by the crystal.
- Can deal with multiple wedges of data and different energy beams (e.g. MAD).
- Models beam as Top-Hat or Gaussian or can use experimental profiles
- Gives information on dose DISTRIBUTION and TIME COURSE, not just average dose.
- Engineered for easy extendibility: can real beam profile now, and actual crystal shape in the future.
- On server: **www.raddo.se** **(!!)**



Zeldin, Gerstel, Garman JAC (2013)

Dose distribution vs exposure strategy with RADDOSE-3D.



0.0001 MGy (grey),
5 MGy (green),
10 MGy (light blue),
20 MGy (dark blue),
30 MGy (red),

20 μm translation

Helical, 0.2 μm/ °

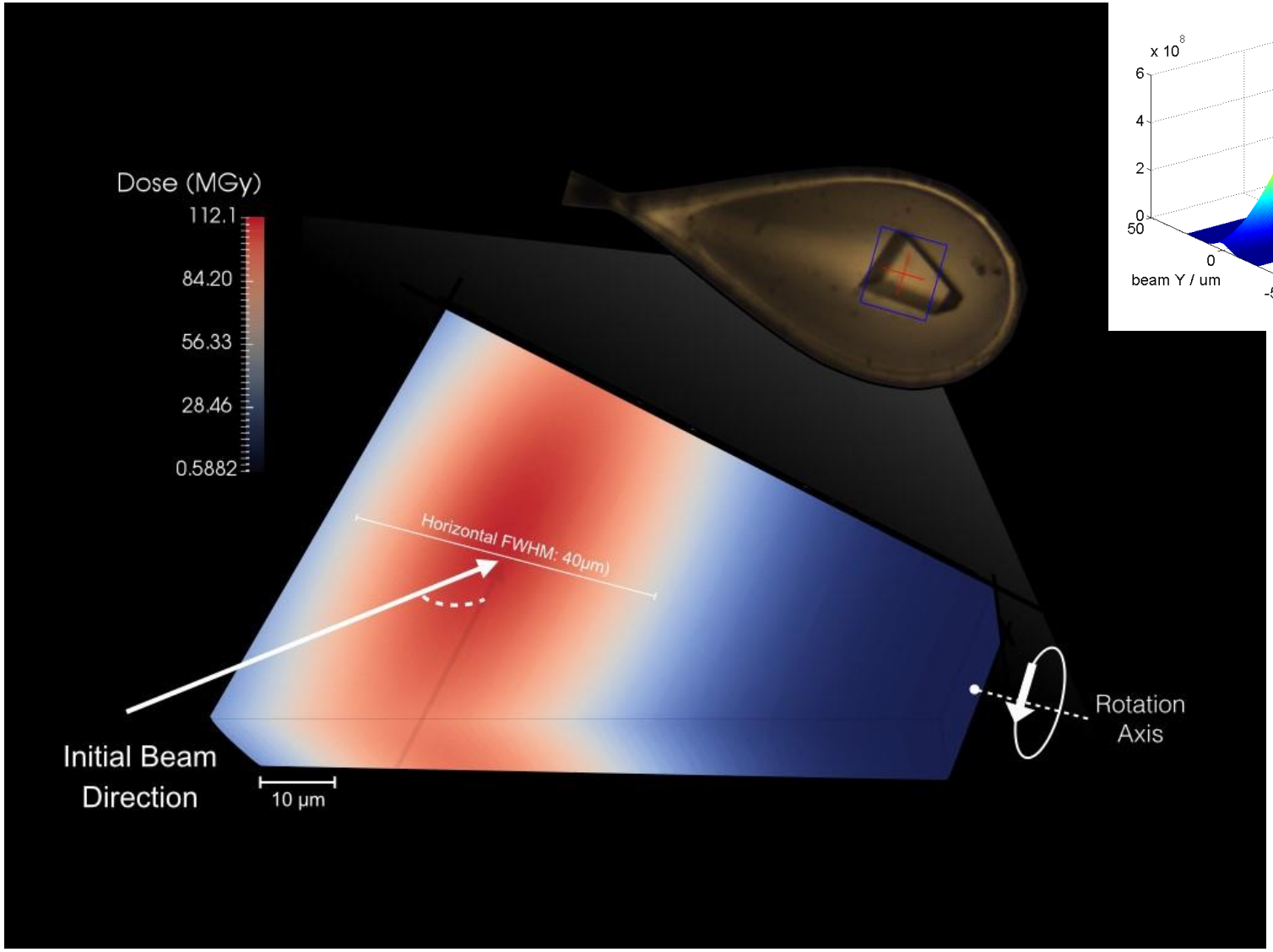
30 μm offset

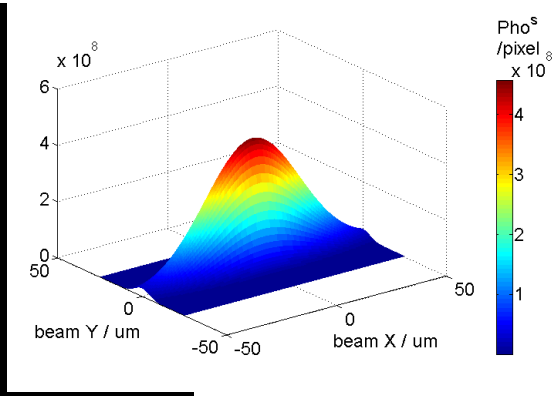
30 μm offset and helical 0.2 μm/ ° .

360° rotation, 100 × 200 × 100 μm^3 crystal
Gaussian beam (FWHM: 20 μm × 20 μm), 12.4 keV, 5×10^{11} ph/s,
1 × 1 mm^2 rectangular collimation: full crystal bathed in beam.

[Bury, Brooks-Bartlett, Walsh, Garman. *Protein Science* (2018)]

Gaussian Beam Profile

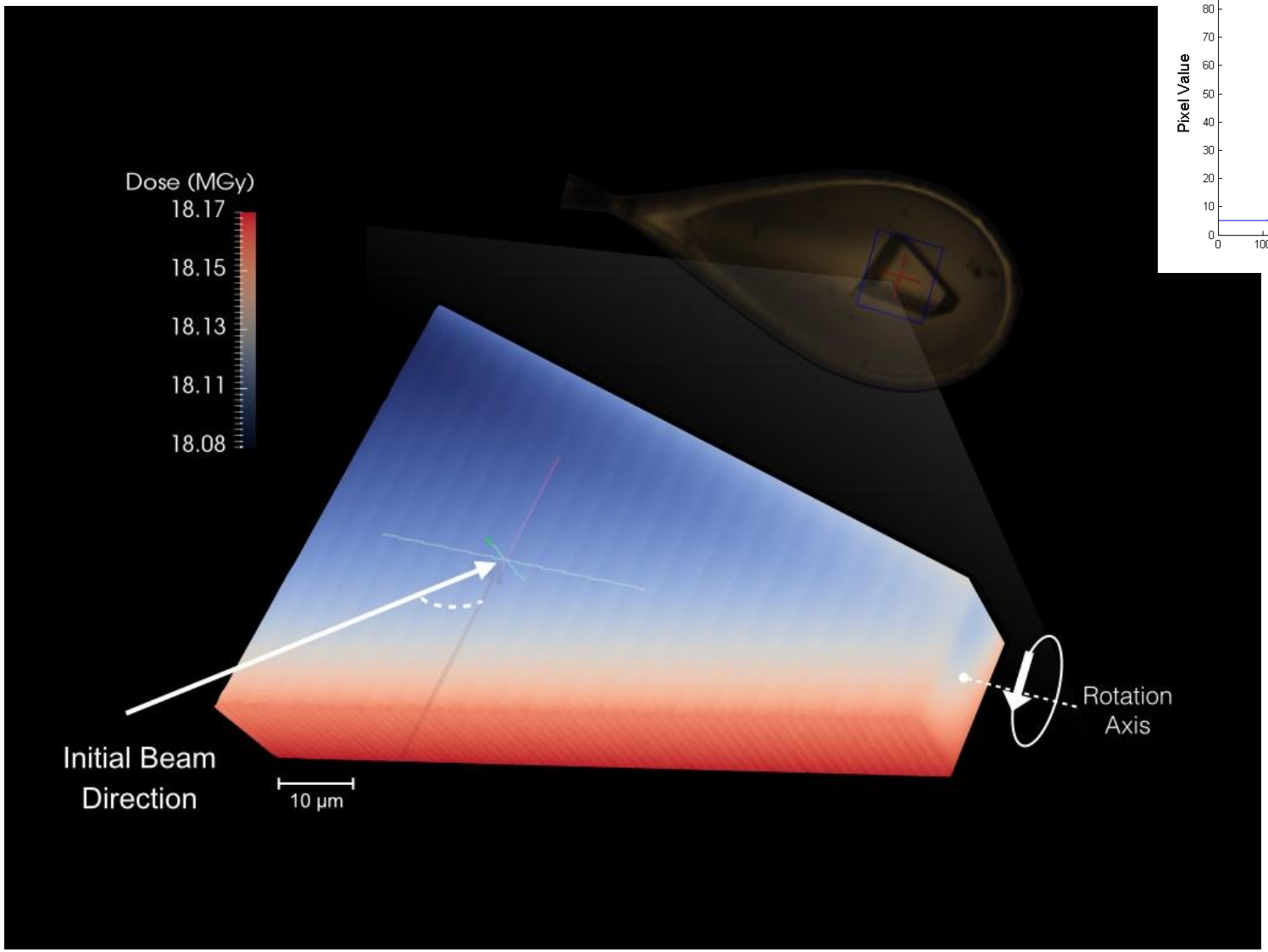


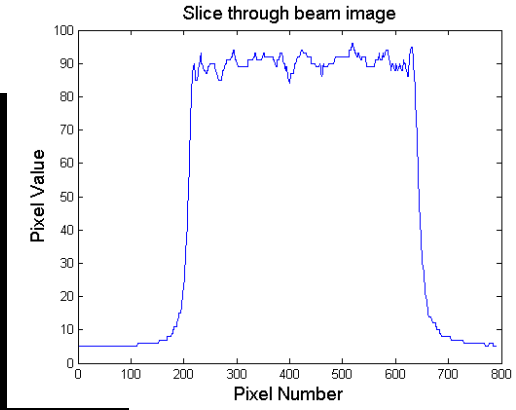


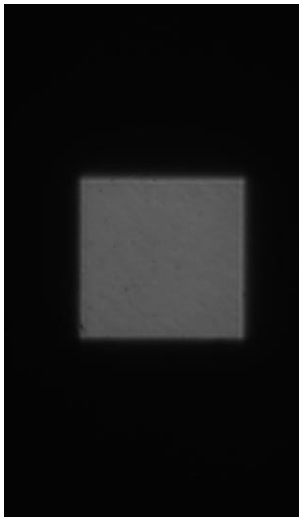
Differential irradiation may lead to differential damage:
get data which merge poorly and are a population of substates

13.2 keV, 60(v)×40μm^2(h) FWHM, 100(h)×160μm^2(v) coll., 5e11 ph/s

Top-Hat profile

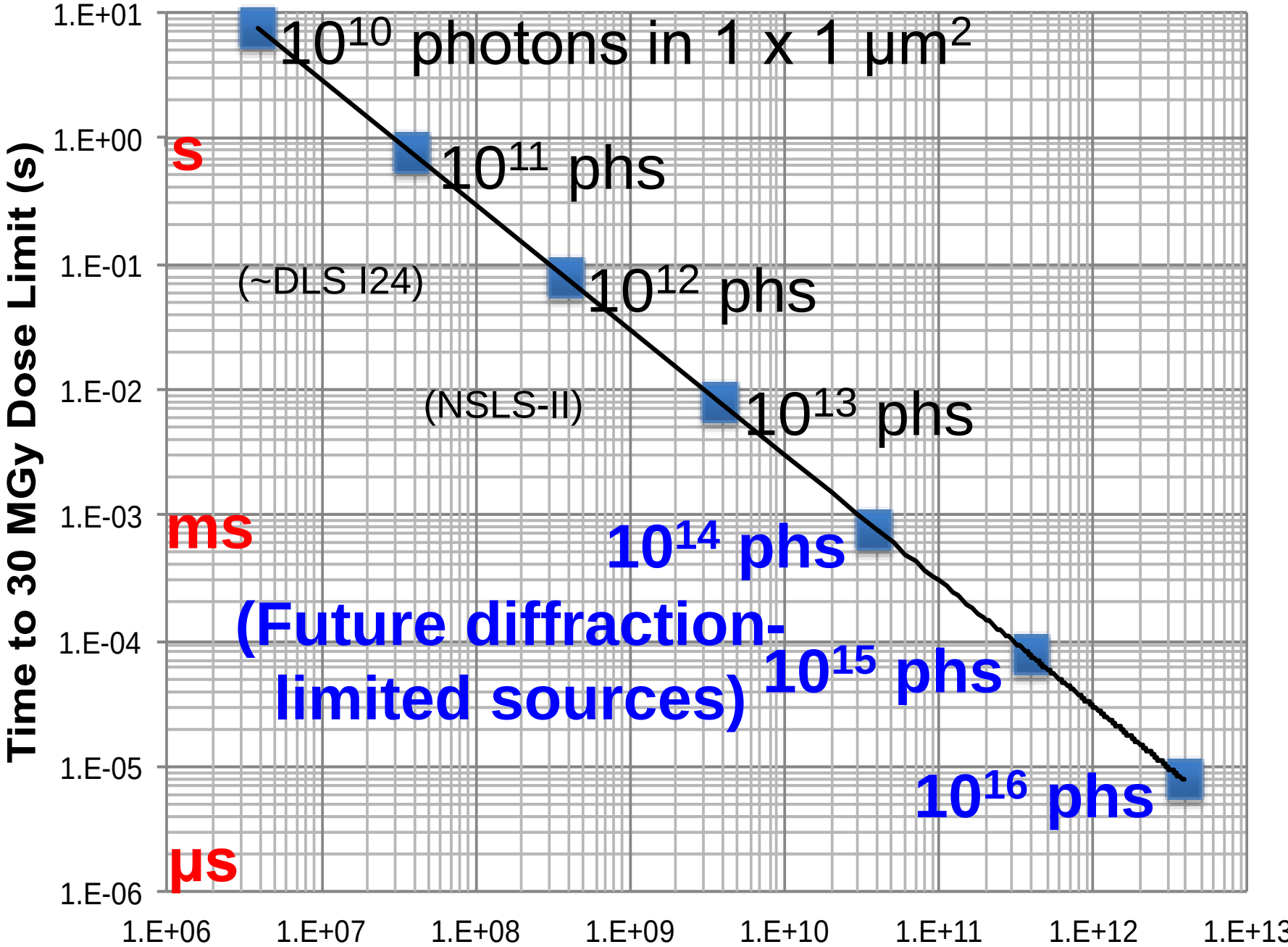






Imaged beam PETRA III, Bourenkov, Schneider

13.2 keV, 100(h) x 160µm^2(v) coll., 5e11 ph/s



[McSweeney using RADDOSE-3D, 2016]

RADDOSE-3D

TEST our new GUI!!

To run RADDOSE-3D for MX, SMX or SAXS (which ever you like!)

Step 1: Download and unzip the RADDOSE-3D GUI from:

https://github.com/jdickerson95/qt_RADDDOSE-3D/releases

There are versions for a PC (Windows_release.zip) and for Linux (Linux_release.zip).

If you have a MAC, there is no new GUI yet, but you can run a limited capability RADDOSE-3D from the WWW site:

raddo.se

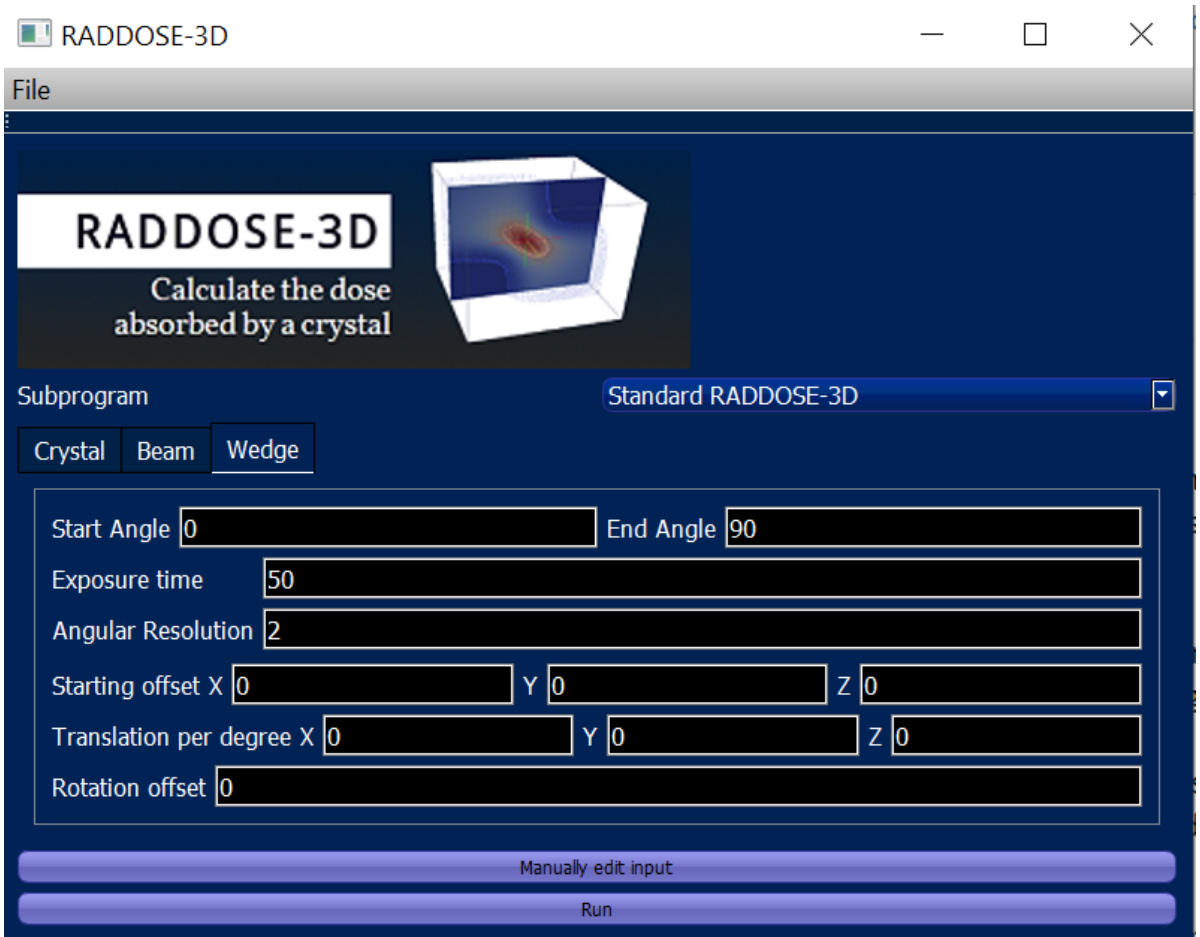
(click on ‘manual interface’ and run the test example first. Then edit the input for a case you would like to try)

To run the GUI you need to have Java installed which you can get free at

https://www.java.com/download/ie_manual.jsp

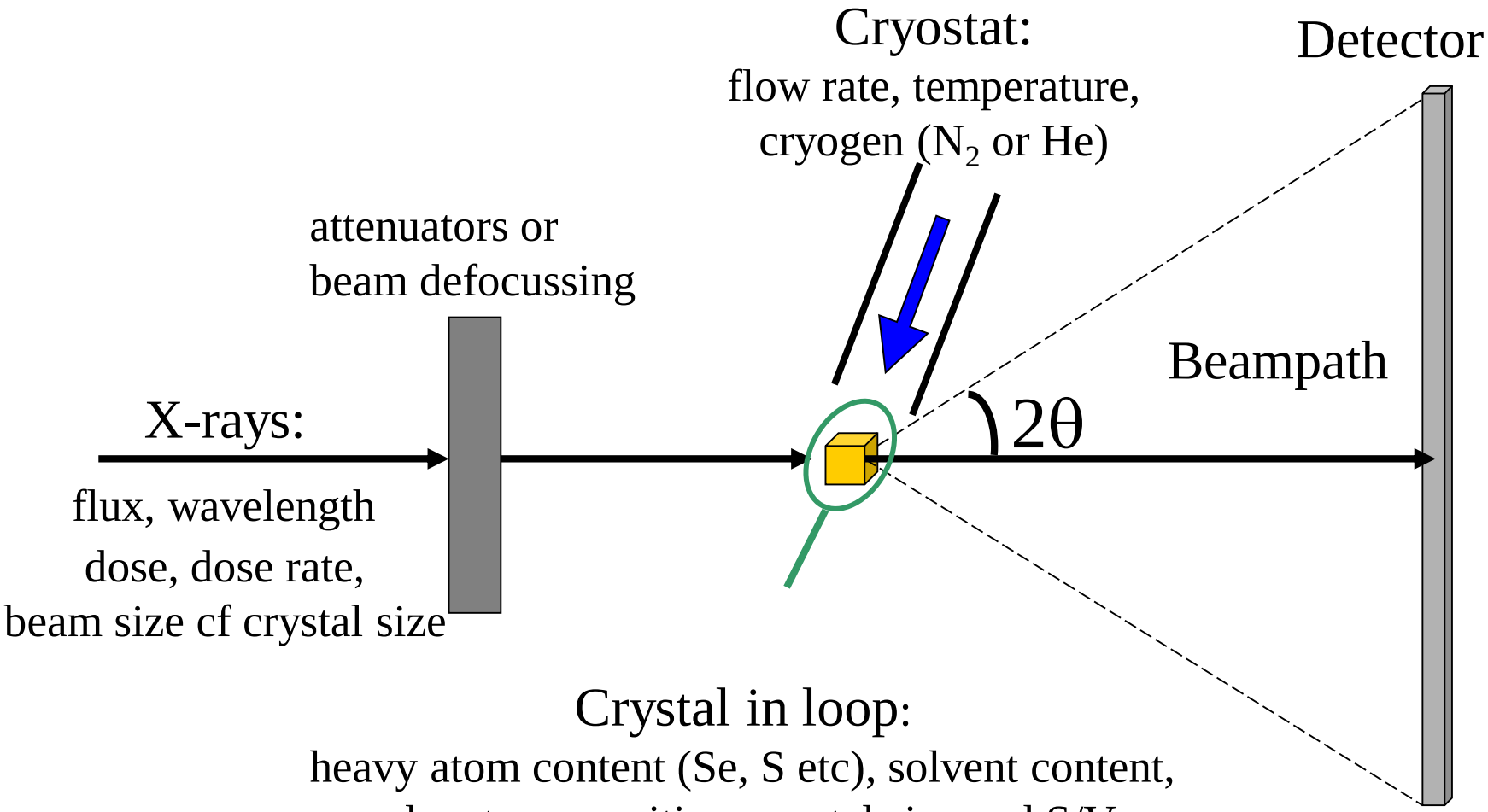
Also, if you have R (https://www.r-project.org/) installed, from the RADDOSE-3D output you will be able to produce 3D representations of the dose distribution in your sample.

Step 3: Find the file RD3D_GUI.EXE and if on a PC click on it. For Linux run it however you usually run executable files. The GUI should open, and you can enter input on 3 tabs: crystal, beam and wedge.



Radiation damage:
The Plan:

- What are the symptoms?
- Why do we care? Effect on MAD/SAD.
- What is it?
- How do we calculate the Dose?
- **What do we know/would like to know?**



Crystal in loop:
heavy atom content (Se, S etc), solvent content,
solvent composition, crystal size and S/V,
amount of residual liquid around crystal,
choice and concentration of cryoprotectant agent,
time in cryobuffer, flash-cooling method (stream or liquid),
cryogen used to flash-cool, amount of crystal manipulation,
humidity, speed of experimenter when flash-cooling.

[Garman, Current Opinion of Structural Biology 2003, **9,** 545-551]

PROBLEM: how do we know that we are making any difference?

- In order to investigate the effects of various parameters on the radiation damage process, we need a robust radiation damage METRIC which is preferably ON-LINE during the diffraction experiment.

No unanimous metric currently/ results from different metrics do not agree.

- Structural changes occur before degradation of diffraction quality is obvious.

Work so far / ongoing:

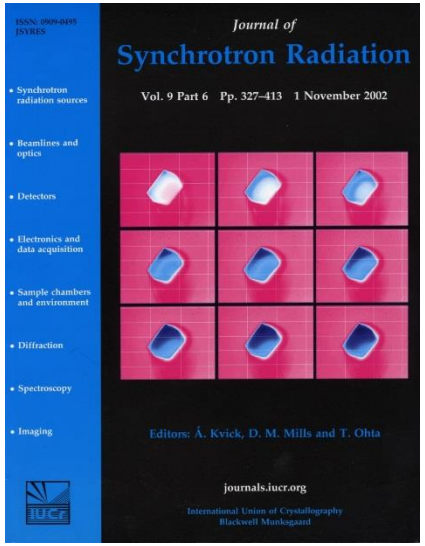
- Lower the cryogen temperature? 40K? 50K? 16K? 140K (no!). Less than a factor of 2 improvement.
- Lower the wavelength? Lots of anecdote + now some systematic results: no effect on damage rate except for small crystals where photoelectron can escape.
- Unit cell expansion as a metric? No!
- Change/ regulate the dose/dose rate regime? No, not at current!
- Effect on MAD/SAD? Order of data collection?
- Minimum crystal size? Several papers (see Holton 2009)
- Beam heating. Not a big factor at current flux densities at cryotemperatures.

Work so far / ongoing:

- X-ray absorption – important parameters defined.
- Remove oxygen? Nothing yet.
- Radiation damage Induced Phasing (RIP)
- Software developments – big progress.
- Add radical scavengers: results disagree.
- Biological implications/applications to mechanistic studies. Now many.
- Room temperature studies: dose rate effects? Results disagree.

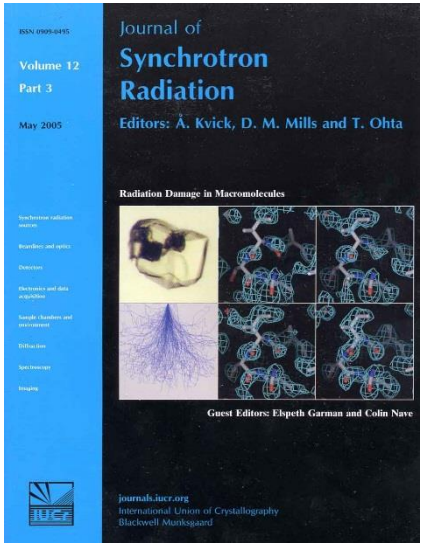
- N.B. Need for **systematic statistically significant** experiments.
- Series of Radiation Damage Workshops

RD2: Dec 2001



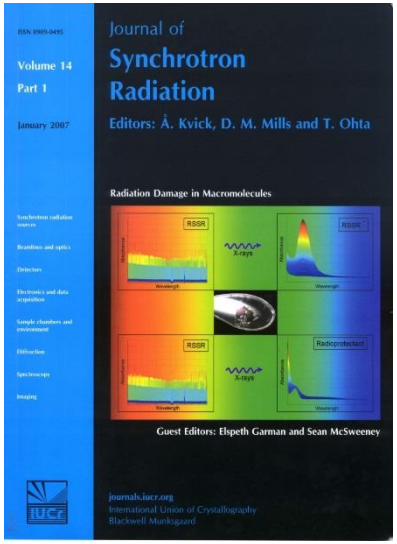
JSR, Nov 2002 (8)

RD3: Nov 2003



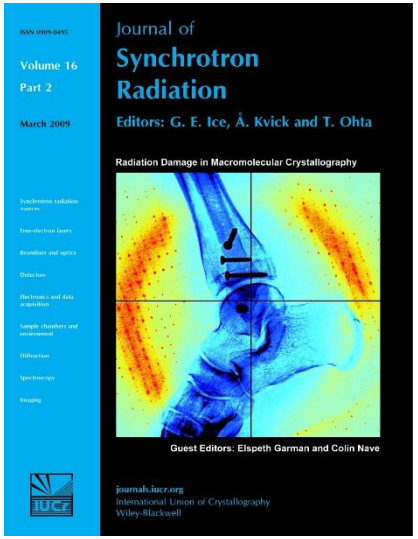
JSR, May 2005 (9)

RD4: March 2006



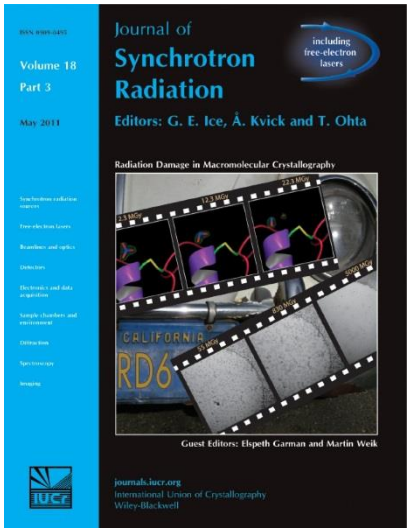
JSR, Jan 2007 (14)

RD5: March 2008



JSR, March 2009 (8)

RD6: Mar 2010

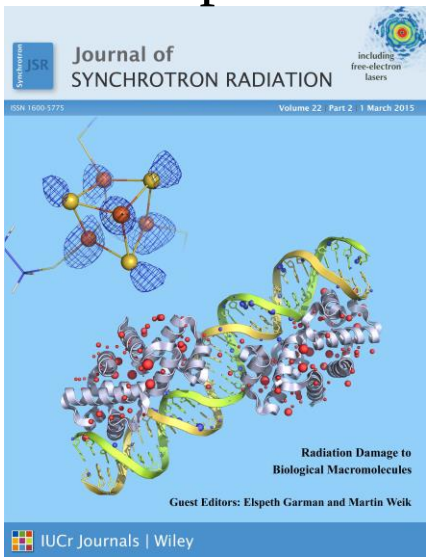


JSR, May 2011 (10)

RD7: Mar 2012

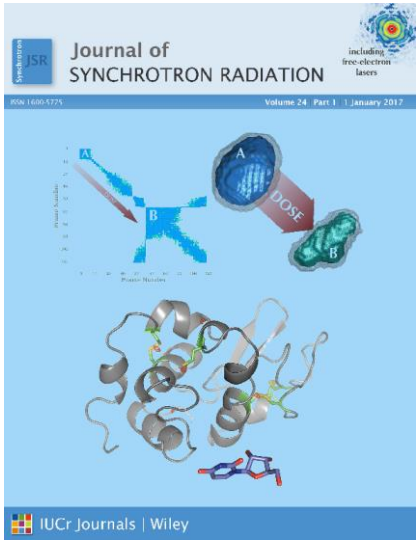
JSR, Jan 2013 (6)

RD8: Apr 2014



JSR, March 2015 (8)

RD9: Mar 2016

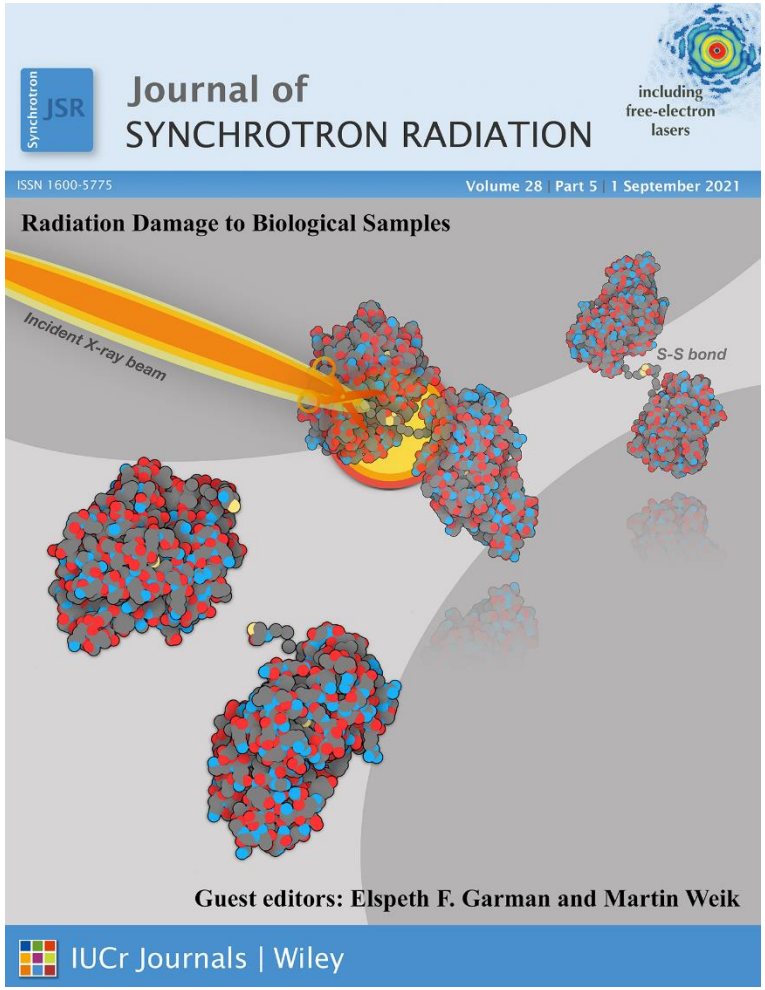


JSR Jan 2017 (8)

RD10: Sep 2018

JSR, July 2019 (9)

RD11: Sep 2021



RD2 to 11:
Total of 94 papers published in JSR Special Issues so far

FURTHER READING:

‘Beginner’s guide to Radiation Damage’
Holton, (2009) *JSR* **16**,133-142

General summary in: Garman, *Acta D* (2010)
66, 339-355

Garman and Weik, Chapter 20 in
‘Protein Crystallography: Methods in Molecular Biology’ (2017) **1607,** 477-489

Summary 1: what can YOU, the experimenter do?

- Do not be afraid to merge data taken from different isomorphous crystals which all had lower doses.
- Back soak non-specifically bound heavier atoms out of your crystals.
- Be ‘absorption aware’ of the contents of your crystal (e.g. Se and buffer) and if possible, avoid cacodylate buffer (arsenic mass=75).
- Match beam size to crystal size

Summary 2: what can YOU, the experimenter do?

- Scavengers: try electron scavengers at 100 K (nitrate/ascorbate/benzoquinone).

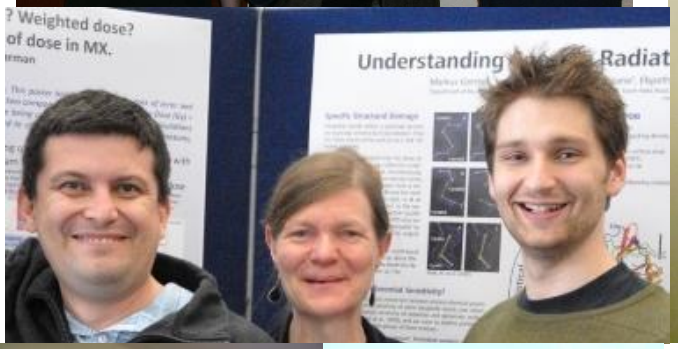
- Dose ‘spreading’: use a tophat profile beam if possible. Consider Helical/Translational data collection.

- So you can estimate the dose, ASK at the beamline:
  - What is the flux today at this energy and with this slit size (‘flux density’)?
  - What is the beam profile today at this beam energy? FWHM in *x* and *y*?

Current status: radiation damage in protein crystals

- Understand a lot more than eighteen years ago, but still not nearly enough…
- Understand how to do experiments better.
- Research has prompted some very exciting new approaches.
- Many complementary methods now being used on the problem in concert with crystallography
- Experiments must involve more than one sample (!) to get statistically significant results: labour intensive and time consuming. Also MUST know incident FLUX density…
- **Radiation damage has DEFINITELY become a mainstream concern**

I thank my past and present group and our collaborators, and acknowledge their huge contribution to the work



Some Garman Group PhD students and Postdocs 2000-2018:
next generation Crystallographers…

diamond

ESRF

Graduate students

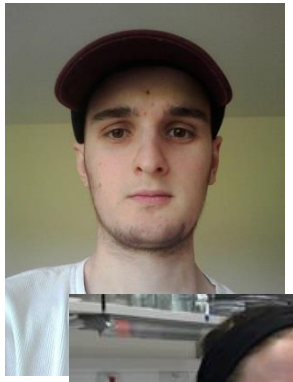
James Murray (Imperial College)
Robin Owen (DLS)
Eugenio de la Mora Lugo (IBS, Grenoble)
Oliver Zeldin (Facebook)
Markus Gerstel (DLS)
Helen Ginn (CFEL, Hamburg)
Jonathan Brooks-Bartlett (Spotify)
Charlie Bury (Medicines Discovery Catapult)

Collaborators:

Raimond Ravelli, Maastricht.
Martin Weik, IBS
Ian Carmichael, NDRL, USA
John McGeehan, Portsmouth
James Holton, UCSF

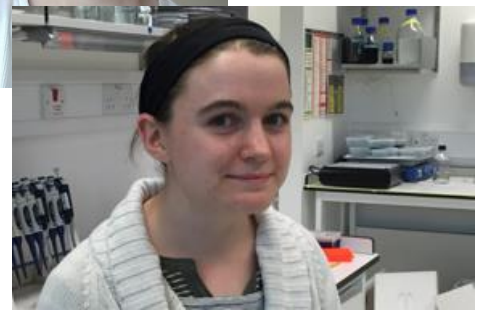
Postdocs

Karthik Paithankar (U. Frankfurt)



Undergraduate Project students

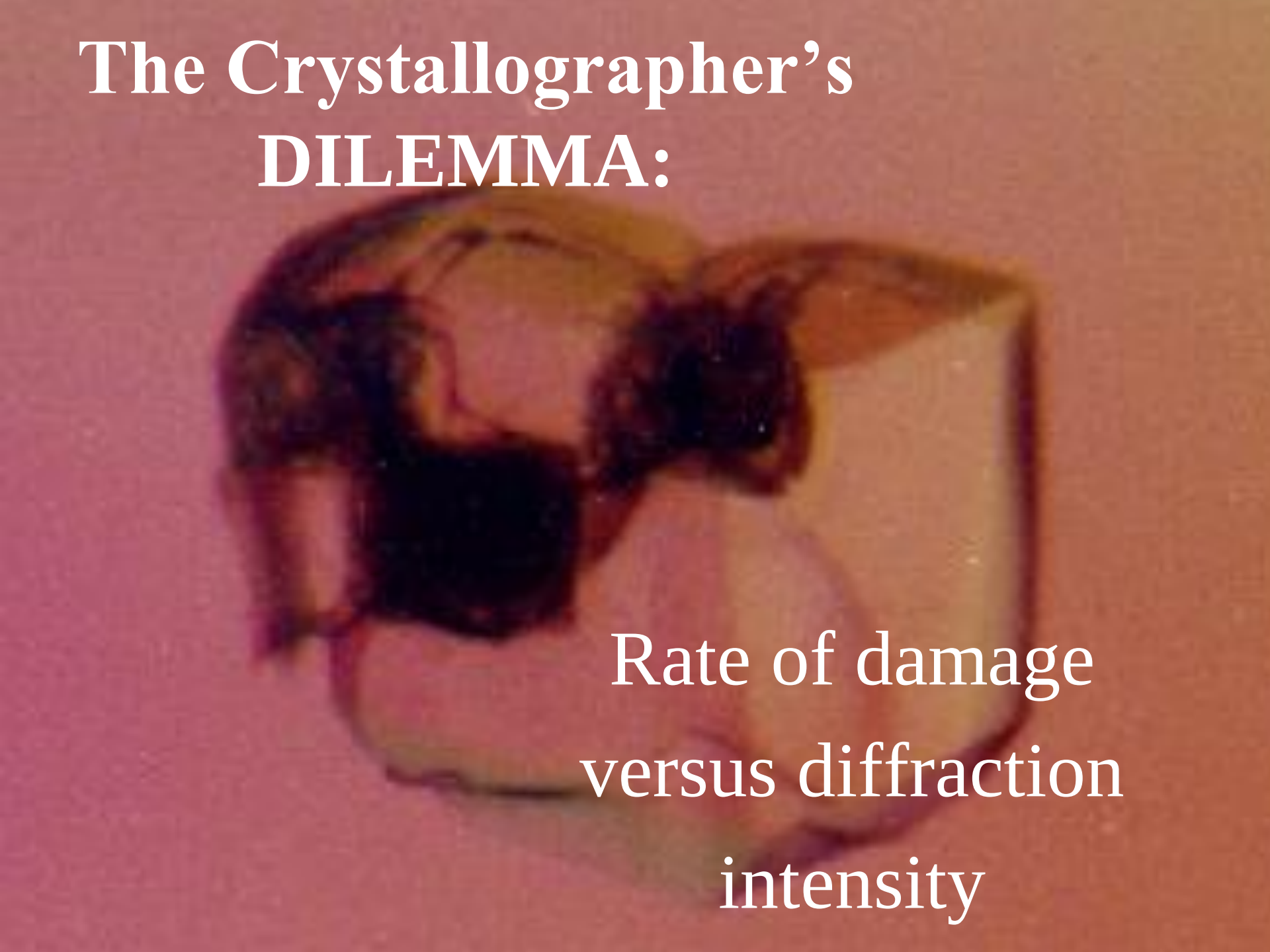
Kathryn Shelley (RABDAM)
Josh Dickerson (RADDOSE-3D)



EPSRC
Engineering and Physical Sciences Research Council
SIXTH FRAMEWORK PROGRAMME
European Commission

Still the first day and my brain is already over full…

The Crystallographer's DILEMMA:



Rate of damage versus diffraction intensity